2014

Circadian Regulation of Mtor Signaling via bmal1 Dependent Mechanism

Rohini Vishal Khapre
Cleveland State University

CIRCADIAN REGULATION OF mTOR SIGNALING VIA BMAL1 DEPENDENT MECHANISM

ROHINI VISHAL KHAPRE

Bachelor of Pharmaceutical Sciences

University of Mumbai

May 2001

submitted in partial fulfillment of requirement for the degree

DOCTOR OF PHILOSOPHY IN REGULATORY BIOLOGY

at the

CLEVELAND STATE UNIVERSITY

March 2014

We hereby approve dissertation of

Rohini Vishal Khapre

Candidate for the Doctor of Philosophy in Regulatory Biology Degree.

This dissertation has been approved

for the Department of

Biological, Geological, and Environmental Sciences

and

CLEVELAND STATE UNIVERSITY

College of Graduate Studies by

Dr. Roman Kondratov, Major Advisor

BGES/CSU **DATE**

Dr. Crystal Weyman, Advisory Committee Member

BGES/CSU **DATE**

Dr. Valentine Boerner, Advisory Committee Member

BGES/CSU **DATE**

Dr. Alexandru Almasan, Advisory Committee Member

Department of Cancer Biology, CCF **DATE**

Dr. Sailen Barik, Internal Examiner

BGES/CSU **DATE**

Dr. Amin Zhou, External Examiner

Department of Chemistry, CSU **DATE**

DEDICATION

I dedicate this dissertation to my husband and daughter. Not only because they helped me succeed but also they have been most affected by my work in the last few years.

First and foremost is my husband, Vishal, without his vision and support, this journey would have not started. Thank you for standing by my side at every step of my way to PhD, so this PhD is for you. We dreamt this doctorate together and made this a reality together. Without your persistence, I would be just a stay home mother. During this long journey, you were my comfort zone, you were with me at every setback or frustrating moments with great patience. Thank you for confiding in me to meet high expectations.

Second is my beautiful princess, RoVija. You are so precious. You were just 2 months old when I started with the Doctorate Program. Since then, your birthdays were my constant reminders of ticking time towards this doctorate. You are the best daughter anyone could ask for. At very early age, you had to understand that “Mommy got to work”, which you did without a single complaint. Thank you RoVa for letting me borrow the “mother-daughter time” for my school. My heart was always filled with guilt every time I dropped you at daycare. I hope, one day, you will realize that my education is an example for you. I will expect no less than a

doctorate from you. Today, every time you say, you want to be a scientist like me, every bit of my time away from you seems totally worth it.

ACKNOWLEDGEMENT

When I started in Doctorate program, I was just a Pharmacy Graduate with a young daughter and minimal knowledge of Molecular Biology. Today, I feel proud just because of my friends and family who lived through this dream with me, supported and guided me.

First and most importantly, Dr. Roman Kondratov. He is the best and kindest advisor I have known. I still remember my first day in Dr. Kondratov's office, he simply burnt all formalities by allowing me to call him "Roman", since then Roman had been the perfect mentor for me and other students. Roman always had faith in me irrespective of my minimal molecular biology background, family responsibilities and a request for avoiding weekend work. I will always be in debt for your guidance. Literally, I fall short of words to express my gratitude towards you. In true sense, you are my Teacher.

Every big building needs a strong foundation, my parents laid down my educational foundation for me. Mom and Dad, you are the best!! Thank you Aaji (my Grandmother-in Law). She was 75, when she travelled from India to help me start with graduate school. She took care of RoVija allowing me to settle in graduate school. Thank you Mama and Atya (my in-laws) for understanding my study schedule and excusing me from many important daughter-in-law duties. Thank you to all my family members, who have always wished best for me, and supported me in this journey.

Thanks to my committee members, Dr. Weyman, Dr. Boerner, and Dr. Almasan. Your valuable advice and guidance has always been invaluable. Dr. Boerner's concept of Trans and Cis mutation and Dr. Weyman's concept of necessary and sufficient are perfectly engraved my brain. I cannot thank enough Dr. Barik and Dr. Zhou for taking on the role of internal and external examiners and giving time from busy schedule to review my PhD dissertation. Thank you Dr. Komar, Dr. Shukla and all BGES faculty for much needed advice and guidance. Special thanks to Dr. Anna Kondratova for her continued guidance and help in various areas that affected my research direction.

Last but not the least, I have the best lab mates in CSU. Yuliya was my first lab mate, and when I started working, she helped me in getting comfortable in the lab. You helped me with basic techniques. Again thanks for being there as a helpful colleague and a good friend. William and I spent maximum time together in lab. Thanks William for your "all things considered" discussions and importantly it was great fun to work with you. Sonal is a perfectionist. I always relied on you for help and I knew that it will be done exactly the way I expected. Nikhil, you really are the man with extra brain. Thanks for letting me borrow the extra one, whenever I needed it most. Kuldeep, Richa, Amol and Shomo have joined the lab recently but feels like they had been with me since the beginning.

I would like to thank all my fellow students who shared fun, helped me with the everyday small problems that I encountered, helped in exam preparations and more.

CIRCADIAN REGULATION OF mTOR SIGNALING PATHWAY VIA BMAL1 DEPENDENT MECHANISM

ROHINI VISHAL KHAPRE

ABSTRACT

Understanding mechanisms of aging is important for the treatment and prevention of age-associated pathologies. However, these mechanisms are not well understood. Recently we have demonstrated that the circadian clock (an internal time keeping system) regulates longevity in mammals, but the molecular mechanisms are not known. The aim of our current study is to investigate a possible interconnection between the circadian clock and mTORC1 (mammalian target of Rapamycin) signaling pathway. mTORC1 pathway is a nutrient response pathway involved in many cellular processes; many recent studies indicate a role of mTORC1 pathway in aging. Here we demonstrate that circadian system regulates mTORC1 signaling *in vivo.* Analysis of liver, heart and spleen from WT mice reveals circadian rhythms in phosphorylation of known mTOR targets such as S6K1 and 4EBPs. These rhythms are disrupted in the tissues of BMAL1 KO mice providing potential mechanistic explanation of reduced longevity of these mice. Further analysis of expression of mTORC1 complex components and upstream regulators demonstrated that some of them have circadian rhythms at protein and mRNA levels. Taken together, these results suggest that circadian

clock controls aging by regulating mTOR signaling pathway through BMAL1 dependent control of gene expression.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

1.1 The circadian clock

Earth's rotation around its axis creates certain environmental changes with a periodicity of approximately 24 hr. Light and dark cycles are one of the most prominent changes which occur approximately every 24 hours. If organisms can keep track of time and anticipate these changes, they can adapt their physiology and behavior to the optimal phase of light and dark cycle. This adaptation can impart survival advantage to organisms (1, 2, 3). For this purpose, many organisms have developed an endogenous time keeping system or device known as the circadian clock (*Circa* = *around, Diem* = *day).* The circadian clock is found in almost all organisms from prokaryotes to eukaryotes. It is represented by an autoregulatory feedback loop of genes generating circadian rhythms in physiological processes (1,4). From Cynobacteria to mammals, several core circadian clock genes responsible for creating circadin rhythms have been

identified. Even though the core circadian genes are not conserved among species, the basic mechanism to create rhythms is conserved. The core circadian clock proteins form an autoregulatory negative feedback loop which works with a periodicity of 24 hr. BMAL1 is a transcription factor and a core component of the mammalian circadian clock. BMAL1 heterodimerizes with another core clock component, CLOCK and binds to E-box elements in the promoter region of target genes to their drive expression (5).

Previously, our lab has demonstrated that *Bmal1* deficiency results in premature aging and age related pathologies in mice. In this work we proposed that BMAL1 is an important regulator of oxidative stress and *Bmal1* deficiency can lead to Stress -induced senescence *in vivo,* which might contribute to premature aging observed in *Bmal1-/-* mice. In agreement with previously published data, we observed that *Bmal1-/-* mice show significantly higher accumulation of senescent cells *in vivo* in comparison with WT. We reported that *Bmal1* deficiency results in increased sensitivity to oxidative stress and Stress -induced senescence. We also investigated the role of circadian clock in mTORC1 signaling pathway. mTORC1 signaling pathway is an evolutionarily conserved nutrient sensing pathway involved in various cellular processes, metabolism and aging. It is activated by nutrients and growth factors. Since nutrients availability depends on organisms feeding rhythms, which are regulated by the circadian clock, we proposed a link between the circadian clock and mTORC1 pathway. We demonstrated that the circadian clock negatively regulates the mTORC1 pathway and disruption of the

circadian clock in BMAL1 deficient mice results in increased mTORC1 signaling, which might contribute to premature aging.

1.2 Characteristics of circadian clock

The circadian clock demonstrates certain characteristics that help to confer selective advantage to organisms.

- It generates rhythms in metabolism and behavior with a periodicity of 24 hours.
- These rhythms are persistent even in the absence of external cues such as light. (in constant darkness)
- Circadian clock can be entrained by external stimuli such as light and food which helps organisms to adapt to environmental changes.
- Circadian rhythms are genetically determined.

In general, the circadian clock system can be illustrated by 3 interconnected components,

1) Input pathway, which receives external cues and transmits them
2) Clock unit, which creates rhythms
3) Downstream pathways, which exhibit these rhythms.

The circadian clock serves to synchronize different biochemical and physiological processes which help many organisms to separate incompatible biochemical reactions. Underlying the importance of circadian clock, circadian rhythms are found at all cellular organization levels.

1.3 Functional organization of circadian clock in mammals.

According to the current model, the mammalian circadian clock is organized in hierarchical manner, with master clock or central oscillator located in the Suprachiasmatic Nucleus (SCN) of hypothalamus region in the brain. Many peripheral clocks exist in different tissues, which are synchronized by the master clock. The SCN is a small collection of around 20000-100000 neurons (in mammals) which receives photic information from retina through Retino-Hypothalamic Tract (RHT). Neurons in SCN show robust circadian rhythms in electrical activity and gene expression. The mechanisms of synchronization of master clock in SCN and peripheral clocks are not clear. However, some studies show that SCN produces humoral and chemical signals that synchronizes peripheral clocks with the environment **(Figure1)**. According to published microarray data, 10% of genes in different tissues are under circadian clock control, which result in rhythmic changes in physiology and behavior.

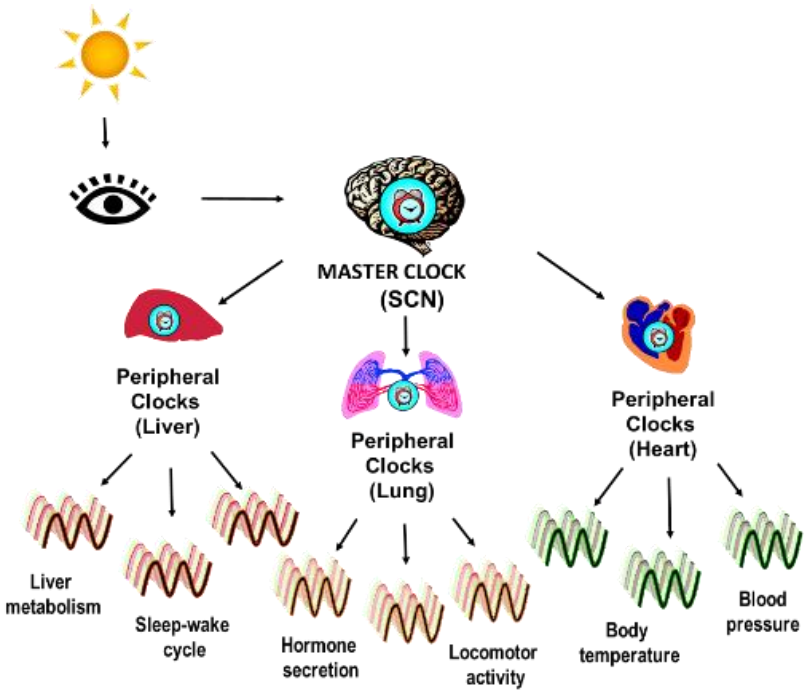


Figure 1 : Functional organization of the mammalian circadian clock

In mammals master clock is situated in SCN (SupraChiasmatic Nucleus) of hypothalamic region of brain. It is entrained by light signals received from retina (eye). SCN creates signals to synchronize peripheral clocks from various tissues to environment. Peripheral clocks create rhythms in various physiological processes.

1.4 Molecular Components of circadian clock

Circadian rhythms are genetically determined and many genes have been identified as a part of the circadian clock. Even though the circadian clock proteins are not conserved among organisms, at molecular level the circadian clock is organized with similar design principles of period, amplitude and phase. It is represented by two interconnected transcription-translation feedback loops.

In mammalian cells, circadian clock oscillations are accomplished by interconnecting auto regulatory feedback loops in which specific clock proteins repress their own transcription and also generate oscillations in transcription of many genes, which are not part of the circadian clock loop. These genes are responsible for producing rhythmicity in processes and behavior (1, 7). Various proteins that comprise the mammalian circadian clock are BMAL1 (Brain Muscle Arnt such as 1) and CLOCK (Circadian Locomotor Output Cycles Kaput), three PERIOD proteins (PER1, PER2, PER3), 2 Cryptochromes (CRY1, CRY2) (7).

BMAL1 and CLOCK are transcription factors and share three characteristic regions involved in their function: one BHLH (basic Helix- Loop- Helix) region and two PAS (Per-Arnt-Sim) region required for DNA binding and heterodimerization **(Figure 2).** BMAL1 and CLOCK heterodimerize in the cytoplasm and the complex translocate to the nucleus, where it binds to E box elements in the promoter region of various genes to drive their transcription. PERIODs and CRYs form the so called positive arm of autoregulatory loop (1, 7). PERIOD has three mammalian homologs Per1, Per2, Per3, containing 2 PAS domains (PAS A and PAS B) involved in PER-PER interactions and CRY binding domain involved in binding with CRYs. In mammals

there are two CRY homologs which are involved in BMAL1: CLOCK repression. PERIODs and CRYs accumulate in the cytoplasm and bind to each other to form heterodimers. These heterodimers translocate back to the nucleus and repress their own transcription by negatively regulating the BMAL1: CLOCK complex, thus forming the negative arm of the loop (1, 7 8, 9) (**Figure 3).**

Another autoregulatory loop for circadian clock is represented by Dec1 and Dec2 (differentially expressed in Chondrocytes) which are bHLH transcription factors. BMAL1: CLOCK complex drives their expression by binding to E-box elements in the promoter region. In feedback, DECs exert a repressing effect on BMAL1: CLOCK induced transactivation of target genes. Two mechanisms have been proposed in their repressing effect: one by physical binding with *Bmal1* through bHLH region and second DECs compete with BMAL1: CLOCK complex for binding to E-box elements in promoter region of target genes (1, 8, 10).

The third auto regulatory loop is represented by REV-ERBs and RORα proteins regulating expression of *Bmal1*. Rev-ERB and ROR proteins belong to retinoic acid-related orphan receptor (ROR) with DNA binding domain recognizing ROR elements (A(A/T)NT(A/G)GGTCA) REV-ERBα and RORα compete with each other for binding to ROR elements in *Bmal1* promoter region. REV-ERBα acts as a negative regulator while RORα acts as a positive regulator of *Bmal1* transcription (1, 8, 11, 12, 13).

In addition to various interconnected autoregulatory loops, additional mechanisms supplement regulation and complexity to circadian clock maintaining Circadian rhythms. Some of these mechanisms include post-transcriptional

modifications, post-translational modifications, chromatin remodeling and stability of Clock proteins and intracellular localization (7).

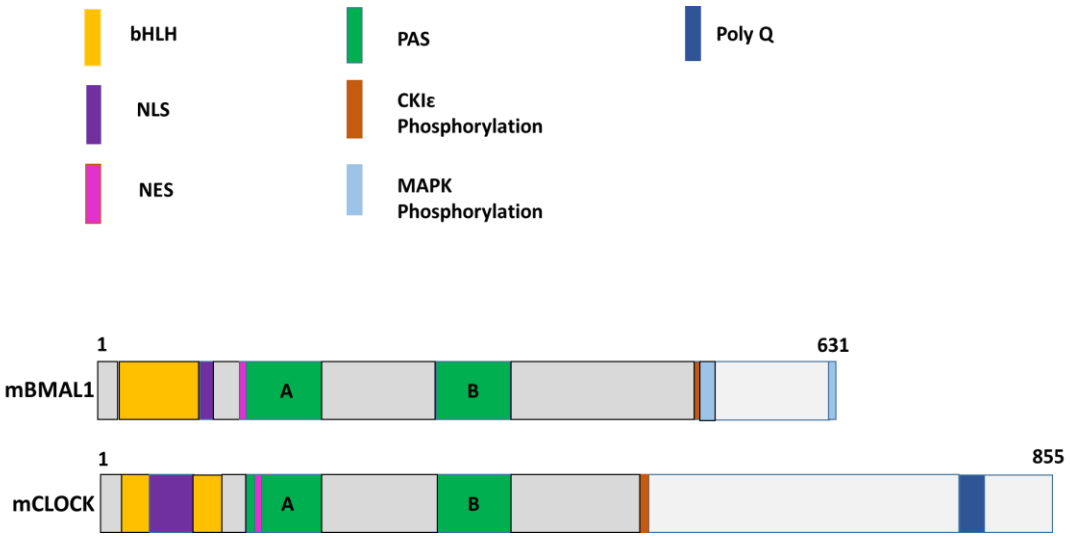


Figure 2 : Functional domains of the mammalian BMAL1 and CLOCK proteins

bHLH: basic Helix loop helix, NLS: Nuclear localization Signal, NES: Nuclear Export Signal, PAS:PER-ARNT-SIM, CKI: Casein kinase I, MAPK: Mitogen activated protein kinase, Poly Q: Poly glutamine motif. This figure is adapted from http://www.sciencedirect.com/science/article/pii/S0959437X05001292

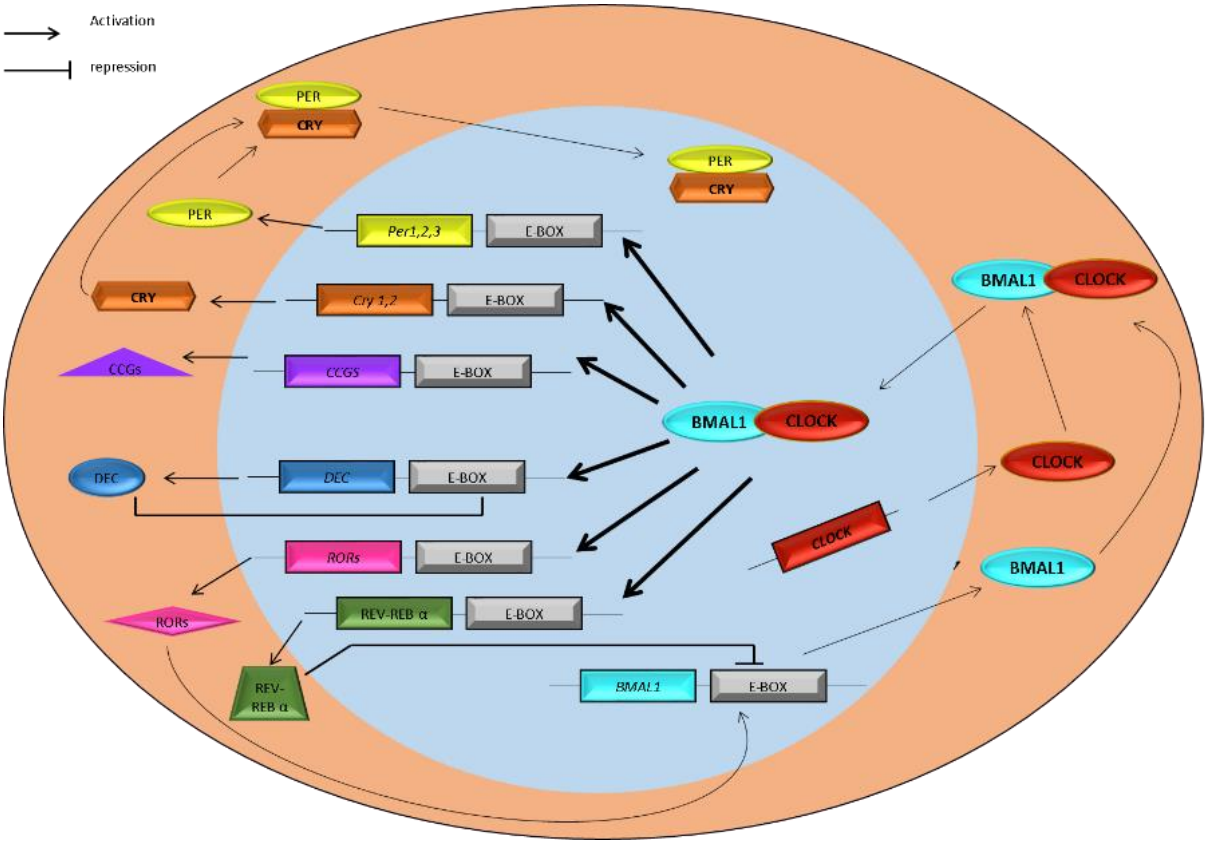


Figure 3 : The positive and negative transcriptional–translational feedback loops controlling the circadian machinery.

BMAL1 and CLOCK proteins form heterodimer complex and drive the expression of the target genes *Pers* , *Crys* , *Decs* , *Rev-Erbα* , *and Rors* through binding to the circadian E-box elements in their promoter region. Upon translation, PERs and CRYs accumulate in the cytoplasm and form a complex which inhibits the BMAL1: CLOCK complex transcriptional activity, and hence their own expression forming autoregulatory negative feedback loop. The nuclear receptor REV-ERB α represses BMAL1 expression and the retinoid-related orphan receptor ROR promotes the transcription of BMAL1 through binding to ROR elements in the Bmal1 promoter. DEC proteins compete with the BMAL1: CLOCK complex for their

own E box located in the DEC promoter and so inhibit their own transcription. The BMAL1: CLOCK complex also regulates the expression of various clock-controlled genes (CCGs), which play important roles in many physiological functions. This figure is adapted from *Khapre et al. Ann Med. 2010 Sep; 42(6):404-15*.

1.5 Disruption of the circadian clock and human health

As the circadian clock regulates various physiological processes, disruption of the circadian clock has an evident effect on physiological processing, disease related pathways and overall well-being of organisms (14, 15). Detailed knowledge about genetic basis of the circadian clock and availability of various model organisms for disrupted circadian clock has linked numerous neurological, cardiovascular and metabolic disorders (8, 14, 15).

The sleep- wake cycle is regulated by the circadian clock. The disruption of clock also affects the sleep-wake cycle leading to Circadian Rhythm Sleep Disorders. (CRSD). Some disorders commonly found in humans are Delayed Sleep Phase Syndrome (DSPS), Advanced Sleep Phase Syndrome (ASPS) and Non-24 advanced sleep syndrome (non-24). Recently, mutations in the human circadian gene *per2* and *ck1δ* have been shown to result in Familial Advanced Sleep Phase Syndrome (FASPS) in humans. In this syndrome, patients exhibit early sleep onset and early awakening. In DSPS, sleep and wake patterns are delayed significantly compared with conventional times and have been linked to mutations in the *per3* gene, whereas individuals with non-24 show 1 hour delay in sleep onset and wake up times every day (17, 18).

Disruption of the circadian clock can affect mood related disorders. Seasonal Affective Disorder (SAD) is one of the mood disorder characterized by depressive symptoms during winter. It is related to desynchronization of environmental and internal body rhythms. Light therapy has been used efficiently

to treat patients with SAD. For individuals with mood disorders, strict daily routines of meal and bed time are beneficial (18).

Many studies have shown that specific mutations of different clock genes show specific behavioral abnormality. Mutation in *clock* gene results in behavior indicative of mania in bipolar disorder in humans. Mice mutant for *per* genes show preference for abusive drugs such as cocaine and alcohol (2, 16). It is known that poor sleep health is related to neuronal disorders. With increased incidences of neurodegenerative disorders such as Alzheimer's disease and Huntington's disease, research for association of disrupted circadian clock has been started recently (2,17).

Approximately 15 % Americans work as shift workers. This rotating work can desynchronize circadian clock at the molecular level in central (SCN) as well as peripheral oscillators and their coordination (16, 18). Mood disorders have also been observed in shift workers. Several studies with night and rotating shift workers have been done.

Many acute and long term studies with shift workers show alternations in carbohydrate and lipid metabolism, growth hormone and glucocorticoid secretion and insulin resistance. Frequency and severity of cardiovascular problems such as hypertension, coronary heart disease, and myocardial infarction is increased in shift workers (8, 16). An increased risk of various cancers such as breast, colon and prostate have been associated with shift work, rapid translocation over time zones and jet lag (1, 16).

1.6 Circadian clock and aging

The purpose of all the studies is to improve and extend human lifespan. However, the question of what controls human lifespan has remain unanswered. To answer this, even though many theories of Aging have been proposed, there is no agreement on this issue. Aging can be considered as either a risk factor for diseases or a consequence of the development of diseases. At molecular level it is represented by a decline in overall fitness and well-being of organism driven by deterioration of systems involved in homeostatic mechanisms (1, 19, 20, 21). With progressing age, circadian oscillations deteriorate with reduction in amplitude. In humans, changes in sleep-wake cycles, hormone rhythms and other behavioral cycles have been described with aging (1, 22). However, in recent years, it has been observed that the circadian clock can equally affect aging and development of many pathological conditions associated with aging.

Development of various models, especially flies and mice with deficiency of different core clock components, has provided a great tool to study the connection between circadian clock and aging (1). Manipulation in light and dark periods resulted in 10% reduction in lifespan of wild type *Drosophila melanogaster (flies).* Another study with mutations in core clock proteins demonstrated reduced longevity in flies. Flies with mutation in period genes showed 15% reduced lifespan compared with WT flies (19, 23).

1.7 Mouse models for disrupted circadian clock and aging

A considerable amount of work has been done on molecular mechanisms of the circadian clock. The mammalian circadian clock has been extensively

studied in mouse models. Experimental models with targeted disruption of core clock genes have added significant input to the knowledge of their circadian and non-circadian function (19). The core circadian clock mutants lose circadian rhythms in gene expression, however the expression levels of many genes change in different ways with each circadian mutant showing unique pattern of clock and clock controlled gene expression, therefore the phenotype for each clock mutant is different (19,20).

1.7.1 *Bmal1-/-* mice

Bmal1-/- mice gives the most severe phenotype among all circadian clock proteins. Bmal1 deficiency results in complete loss of circadian rhythms in gene expression and activity (1, 20, 19).

Our lab is working with *Bmal1-/-* mice. The most prominent phenotype observed in this mice is a premature aging. Previously, our lab has reported that *Bmal1-/-* mice are indistinguishable from WT littermates at birth but start to show signs of growth retardation and premature aging at 16-18 weeks of age (4-5 months). At age of 35-40 weeks, *Bmal1-/-* mice look like aged mice (6, 19, and 20). The average lifespan of Bmal1 deficient mice is 37.0 $\pm$ 12.1 weeks while average life span of WT mice with same background is approximately 120 weeks (6).

Along with reduced lifespan, *Bmal1-/-* mice also display many age related pathological conditions as follows:

- Reduced organ weight (kidney, spleen)

- Sarcopenia (reduced muscle mass associated with decrease number of muscle fibers and their diameter)
- Osteoporosis (reduced bone mass and composition)
- Reduced visceral and subcutaneous adipose tissue
- Decreased hair growth
- Development of cataract
- Chronic cornea inflammation
- Changes in blood cell composition
- Ectopic calcification of tendons and cartilage
- Male and female infertility

Data published from our lab and different groups indicate that Bmal1 plays a significant role in normal tissue homeostasis (19, 20, 21, 22, and 23). The molecular mechanisms involved in premature aging in *Bmal1* deficient mice are not known. Our lab is working to identify these mechanisms and one of the potential mechanism suggested was regulation of ROS (reactive oxygen species) homeostasis. *Bmal1* deficient mice displayed increased accumulation of ROS in the tissues (heart, kidney and spleen), which also demonstrated age dependent reduction in size. Our lab demonstrated that treatment with antioxidant N-acetyl-L-cysteine to *Bmal1* deficient mice through their entire lifespan significantly increased average and maximum life span in mice. It also reduced certain age related pathologies such as cataract and weight loss but had no effect on many other age related pathologies observed in *Bmal1* deficient mice. This indicated that

oxidative stress by ROS can affect aging but other ROS independent mechanisms might be also involved (21).

1.7.2 *Period 1, 2 -/- mice*

Deficiency of Period 1 and Period2 proteins result in lack of circadian rhythms in behavior and gene expression. (19, 24, 25). Period 2 deficiency increases genetic and radiation induced cancer susceptibility. Overexpression of Period 1 and period2 has shown to reduce tumor growth and promote apoptosis in vivo and in vitro (20, 26, 27, 28).It is observed that Period 1and 2 are downregulated in various human tumors. Therefore *Period 1* and *2* are considered as tumor suppressor genes (20) *Period 1* and *2* deficient mice also show certain features of premature aging such as faster decline in fertility, loss of soft tissues and kyphosis at 12-14 months of age (19, 25-29).

1.7.3 *Cry 1, 2 -/- mice*

Deficiency of *Cry1* and *Cry 2* also results in arrhythmic behavior. In contrary to the role of Period genes in tumor suppression, *cry1* and *2* deficiency in p53 deficient mice resulted in increased lifespan and resistance to cancer. It has been shown that deficiency of *Cry1* and *2* promotes apoptosis after UV irradiation (20, 30, 31, and 32). *Cry1* deficient mice show reduced life span (our unpublished data).

1.7.4 *Clock -/- mice*

Recently our lab has shown that *Clock* deficient mice show a 15% reduced average lifespan with increased incidences of cataract and dermatitis. These mice have normal body weight and organ weights (20, 33). Other labs have shown that

Clock deficient mice do show arrhythmicity in peripheral tissues with period slightly shorter than 24 hr (20, 35, and 36).

It has been reported that deficiency of CLOCK and its paralog NPAS2 (double mutant) results in arrhythmicity in behavior both in central and peripheral oscillators. Mice display reduced life span and body weight and are sterile (20, 37-40).

All the circadian clock mutant mice are considered as models of disrupted circadian clock and we can expect each mutant to display abnormalities associated with general dysfunction of circadian clock. However, as discussed above, pathological conditions associated with each clock mutant are different. This indicates that circadian proteins might have a unique role in tissue homeostasis, which is independent of clock. Different phenotypes of circadian clock mutants suggests that deficiency of specific clock gene might demonstrate specific phenotype in addition to general abnormalities due to disruption of the circadian clock.

1.8 Senescence and aging

Accumulation of senescent cells in tissues has been observed with aging. It is believed that senescent cells can contribute to age-related pathologies (2, 41). Senescence (derived from the Latin word *Sensex* means old) is a specific metabolic state of cells characterized by certain changes described below. It was first described by Heyflick and Moorhead demonstrating that after definite number of divisions, embryonic derived human fibroblasts enter irreversible growth arrest

displaying limited proliferative potential *in vitro*. This is known as the Heyflick limit (2, 42, and 43). Other than humans, senescence is also demonstrated in other mammalian species and also in chicken cell cultures. Senescence also occurs in other cell types such as keratinocytes, glial cells, endothelial cells, vascular smooth muscle cells, lymphocytes and lens cells (42, 44-50).

Senescent cells display the following specific characteristics:

- **Irreversible growth arrest**: indicates limited proliferative potential and once the growth is arrested, it cannot be stimulated to reenter the cell cycle (43,51).
- **Metabolically active**: It is demonstrated that senescent cells secrete various factors that can affect tissue physiology (44)
- **Resistant to apoptosis**: Senescent cells have been shown to resist apoptosis and can remain viable for indefinite period(52,53)
- **Enlarged and flattened morphology:** Senescent cells are enlarged and flattened. They also show an increase in the size of the nucleus and nucleoli, along with an increase in the number of Golgi and lysosomes and appearance of vacuoles in cytoplasm and endoplasmic reticulum (54).
- **Changes in the expression or activity of proteins:** Proteins showing altered expression or activity in senescent cells are known as biomarkers of senescence. One of the commonly used biomarkers is SA-β Gal (Senescence associated β-galactosidase activity). β-galactosidase is a lysosomal hydrolase usually active at pH 4. Only in senescent cells and not in quiescent or non-senescent cells, β-galactosidase activity can also be

detected at pH6. There are other proteins such as matrix degrading proteases, chemokines and cytokines expressed in fibroblasts at senescent stage (55, 56, 57).

Senescence can be divided into three different types, 1) Replicative, which results from limited proliferation of proliferating cells. It is considered to be due to telomere shortening. 2) Stress induced or premature senescence, which can occur due to oxidative stress or genotoxic stress from DNA damage. 3) Oncogene Induced senescence is considered as protective mechanism to avoid cell proliferation **(Figure 4)** (58-60)

Previous studies based on cells explanted from old donors showed fewer population doubling than young donors, indicating that cells have an intrinsic counting mechanism. This mechanism limits cell proliferative potential after a definite number of doubling times. However, the role of replicative senescence has not been established *in vivo*. With extensive research in the field, senescence is believed to be a cellular state that can be induced by extrinsic factors such as various stresses and these factors also affect cellular intrinsic mechanism (61, 63, 64, 65). It is also believed that stress-induced senescence is a tumor suppressor mechanism however, new role of stress-induced senescence can contribute to age associated senescence (2, 61).

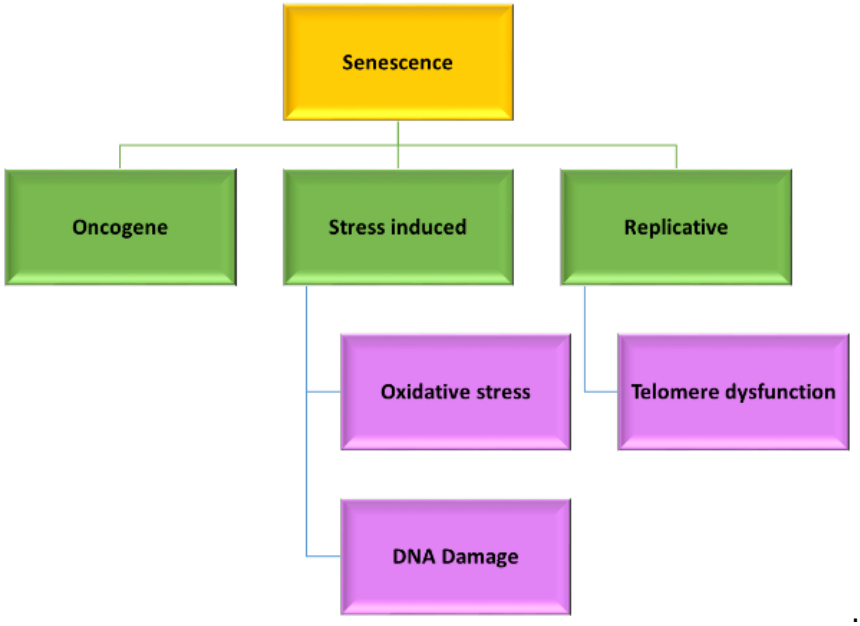


Figure 4 : Different types of senescence

Senescence can be divied into 3 types :1) replicative, occuring due to telomere dyfunction and loss of proliferative potential 2) Stress induced occurs due to oxidative or genotoxic stress and 3) Oncogene induced,whichoccurs due to oncogene activation and acts as a protective mechanism.

1.9 mTOR signaling pathway, metabolism, and aging

TOR (Target of Rapamycin) signaling pathway is an evolutionarily conserved nutrient sensing pathway found from budding yeast to humans. As the name suggests, it was discovered as a target of Rapamycin. Rapamycin was discovered as an antifungal agent, produced by a strain *Streptomyces hygroscopicus* found in soil samples of Polynesian island Rapa Nui. Rapamycin was later recognized for its immunosuppressant and anti-cancer activity (66, 67, 68, and 69). The physiological significance of mTOR is associated with its central role in regulating cellular processes in response to nutrients, growth factors, change in energy status, and stress conditions such as hypoxia. Its central role in metabolism makes mTORC1 crucial for normal growth and development. mTOR has been liked with many diseases such as cancer, metabolic diseases and aging (68-73).

1.10 mTOR and Cancer

As a regulator of cell growth and proliferation, mTOR plays an important role in cancer. Mutations in *Tsc1* or *Tsc2,* upstream inhibitors of mTORC1 result in hamartomatous syndrome (multiple benign and malignant tumors) indicating link between mTOR and cancer (74, 75)

PI3K/AKT signaling activates mTORC1. Oncogenic transformation by hyper activation of AKT signaling is mostly mediated by mTORC1 (76, 77). Dysregulated protein synthesis has been reported in cancer. 4EBPs (inhibitor of eIF4E) is a direct and eIF4B is an indirect target of mTORC1. Both proteins regulate the rate

of cap-dependent translation initiation. (72, 78). The concept of using Rapamycin (mTOR inhibitor) analogs for cancer treatment is growing. Rapamycin and its analogs, in combination with chemotherapy, are in clinical trials (79-80).

1.11 mTOR and metabolic diseases

Being a nutrients sensor, mTOR acts as a major regulator of metabolism. Under diverse nutrient conditions, mTORC1 downstream targets mediate different effects. Recent studies showed that excess nutrients results in insulin resistance and type II diabetes probably via mTORC1 target S6K1 (81, 82, 83, and 84). Studies with S6K1 deficient mice demonstrates its importance in growth development. S6K1 deficient mice are smaller at birth as compared with WT mice. They also demonstrate Hypoinsulinemia and reduced white adipose tissue due to reduced β and adipocyte cell size respectively (85).

1.12 mTOR and senescence

Increased cell size is a hallmark of senescence. mTORC1 is a major regulator of cell size and plays a critical role in cellular senescence. Rapamycin treatment has been shown to delay or inhibit cellular senescence. Another study with a mice model with constitutive Wnt signaling in epidermal compartment, showed mTOR dependent rapid growth of hair follicles followed by senescence that was inhibited by Rapamycin (86).

1.13 mTOR and aging

The role of mTOR in aging is like a double-edged sword. It is critical for growth and development but could be detrimental during late stages of life. Many

aging studies have shown that decreased mTOR activity leads to extended lifespan. In *C.elegans* and *Drosophila*, mutations with mTOR pathway genes have extended longevity (87, 88). Decrease in TOR signaling by low dose of Rapamycin lead to extended chronological life span of yeast (89). Interestingly, long lived Dwarf mice show downregulation of mTORC1 effectors in liver and skeletal muscle (90). The S6K1 deficient mice model is believed to a mimic food restriction model having extended life span and with females displaying increased a longevity, with less age related pathologies (91). Another study with *Drosophila* showed that overexpression of d4EBP results in increased longevity (92).To summarize, studies with inhibition of mTOR and its downstream targets show that mTOR plays a critical role in regulation of life span.

1.14 mTOR kinase

mTOR (mammalian target of Rapamycin, also known as FRAP, RAFT, RAPT) is a 289 kDa serine/threonine protein kinase and belongs to a family of PIK-related kinases (phosphatidylinositol kinase-related kinases). Members of this family share strong homology of their C-terminal domain with catalytic domain of PI3K (phosphatidylinositol 3-kinase). Other members of this family include ATM, ATR, and RAD 53 (93, 94, 95).

1.15 Domains of mTOR

mTOR contains many functional domains involved in its function **(Figure 5)**. At its N terminus, mTOR contains 20 tandem HEAT repeats. HEAT is abbreviated from Huntington, elongation factor 3, the **A** subunit of type 2A phosphatase

(PP2A), **T**OR. These HEAT motifs are believed to mediate protein-protein interactions.

As mentioned before, C terminal contains a kinase domain that is highly conserved and displays serine/threonine kinase activity. It contain around 300 residues region from amino acids 2115 to2446 (96, 97, and 98).

To the N-terminal of kinase domain, mTOR contains a regulatory domain of approximately 100 amino acids (2015-2114 in human mTOR) known as FRB (**F**KBP12-**R**apamycin **B**inding domain). FKBP12 also known as the FK506 binding protein is an immunosuppressant binding protein. The crystal structure of FKBP12-rapamycin bound to FRB domain shows that Rapamycin simultaneously binds to the FRB domain and FKBP12 and inhibits mTOR. A mutation of the conserved Serine residue at aa2035 was shown to confer resistance to Rapamycin (97, 99).

FAT (**F**RAP, **ATM**, **T**RRAP) is another domain in mTOR located at the N terminal of FRB and Kinase domain. It is around 500 amino acids and spans approximately 1513-2014 aa in human mTOR. Crystal structure shows that it contains 3 domains (TRD1, TRD2, TRD3) from TRP repeat family and one HEAT family domain (HRD). Although the function of the FAT domain is not clear, it was proposed to be involved in protein –protein interactions. New crystal structure of mTOR shows FAT domain clamping on kinase domain, illustrating its importance.

FATC (**F**RAP, **A**TM, **T**RRAP, **C**arboxy terminus) is a 35 amino acids long domain found at the extreme C terminus in mTOR. It usually occurs with FAT domain and might work with FAT in catalytic function in PIK-related kinases (97, 99,100).

Between the kinase and FATC domain is a domain from amino acids 2427-2516 known as FIT (**F**ound **i**n **T**OR) domain. It is also known as a regulatory domain due to presence of 3 phosphorylation sites, T2446, S2448, S2481(97).

1.16 Phosphorylation sites of mTOR

There are 4 important phosphorylation sites in mTOR involved in its regulation.

1. T2446, found in the FIT domain that is probably regulated by AMPK depending on nutrient availability.
2. S2448, found in the FIT domain and phosphorylated by its own target S6K1, depending on amino acid and nutrient status.
3. S2481, found in the FIT domain that is autophosphorylated. This phosphorylation might be promoted by insulin signals.
4. S1261, found in the HEAT domain is phosphorylated by insulin/PI3-K in amino acid dependent, Rapamycin insensitive and autophosphoryation-independent manner (97,100,101).

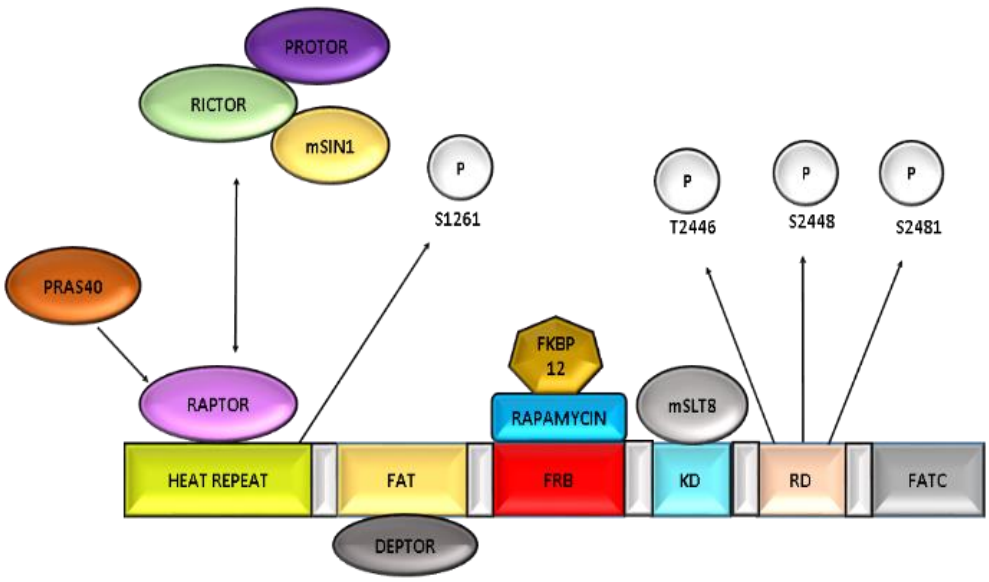


Figure 5 : Schematic representation of mTOR domains, interacting proteins and known phosphorylation sites.

HEAT: Huntington elongation factor 3, PR65/A, TOR FAT: FRAP ATM TRRAP, FRB: FKBP12 Rapamycin binding, KD: kinase domain, RD: Regulatory domain, FATC: FRAP ATM TRRAP carboxyl terminus

1.17 mTOR complexes

mTOR exists in two structurally and functionally distinct complexes: mTORC1 and mTORC2. They were identified based on sensitivity to Rapamycin, with mTORC1 being sensitive and mTORC2 Rapamycin insensitive. However, recent data suggests that mTORC2 also shows sensitivity to Rapamycin after prolonged treatment. mTORC1 and mTORC2 phosphorylate different target proteins (S6K1 and 4EBP1 by mTORC1, AKT, and SGK1 by mTORC2) whose phosphorylation is used as a readout for their activity.

mTORC1 regulates various cellular processes such as protein synthesis, autophagy, metabolism, cell size, and cell proliferation. Compared to mTORC1, the role of mTORC2 is poorly understood (102,103).

1.17.1 mTORC1 complex

In addition to mTOR, mTORC1 complex consist of four other components: Raptor, mLST8, Pras40, and Deptor. Raptor (**R**egulatory-**a**ssociated **p**rotein of m**TOR**) is a 150 KDa mTOR-binding protein involved in recruitment of mTOR substrates for phosphorylation. Raptor interacts with the N-terminal HEAT domain of mTOR for binding (104).

mLST8 (**m**ammalian homolog of **l**ethal with **s**ec-13 gene 8), also known as GβL (G protein β-subunit –like protein) is a 36 KDa protein that is a part of mTORC1 and mTORC2 complex. It binds mTOR in the kinase domain but its role is not clear. Recent data suggest that mSLT8 acts as a positive regulator of mTORC1; its deletion has no effect on mTORC1 activity (105).

PRAS40 (**P**roline-**R**ich **A**KT **S**ubstrate 40 kDa) is a 40 kDa protein which acts as a negative regulator and physiological substrate of mTORC1. It binds to mTOR at its kinase domain. It also binds to Raptor using TOS like motif and competes with mTORC1 substrates for Raptor substrate binding sites. In response to insulin, AKT phosphorylates PRAS40 at T246 which facilitates its phosphorylation by mTORC1 on S183. Phosphorylation by AKT and mTORC1 results in dissociation of PRAS40 from mTORC1, resulting in activation of mTORC1 (106).

DEPTOR (**DEP** domain containing m**TOR** interacting protein) is a 48 kDa protein, which was recently identified as a negative regulator of mTOR. It is part of both mTORC1 and mTORC2 complexes. DEPTOR has two domains a N terminal DEP (**D**isheveled, **E**gl-10, **P**leckstrin) and a C-terminal PDZ (**P**ostsynaptic density 95, **D**iscs large, **Z**onula occludens-1). DEPTOR specifically interacts with mTOR at the C terminal portion upstream of kinase domain via the PDZ domain. DEPTOR doesn't react with mLST8, which is a common component of both complexes. Downregulation of DEPTOR by shRNA results in increased mTORC1 and mTORC2 activity, as indicated by phosphorylation of their specific targets (107,108).

Effectors of mTORC1

mTORC1 phosphorylates many downstream targets. The two best known mTORC1 substrates are S6 kinase 1 and 4EBP proteins. S6K1 is an AGC family member that is encoded by *s6k1* gene. Out of 7 known phosphorylation sites, mTORC1 specifically phosphorylates S6K1 at T389, which is present in the

hydrophobic region and is important for stimulation of S6K1activity. Activated S6K1 promotes mRNA translation through various substrates such as ribosomal protein S6, SKAR, eEF-2K, eIF4B and PDCD4. S6K1 phosphorylates ribosomal protein S6 on four phosphorylation sites, S235, S236, S240 and S244. Phosphorylation of S6 at the mentioned four sites is also used as readout for mTORC1 activity (77,109,110).

4EBPs (eIF4E- binding proteins) are translation repressors consisting of 3 proteins 4EBP1, 4EBP2 and 4EBP3 that are encoded by three different genes. Though all three forms are phosphorylated by mTORC1, 4EBP1 is the most studied form. Hypo phosphorylated 4EBPs have high affinity for eIF4E and their binding prevents its interaction with eIF4G required for Cap-dependent protein translation initiation. Phosphorylation of 4EBPs result in dissociation from eIF4E. Out of four known phosphorylation sites, mTORC1 have been shown to phosphorylate 4EBPs on two sites (T37, T46) *in vitro* which is required for further phosphorylation on T70 and S65 in a hierarchical manner. We have used antibodies against phosphorylation at Thr37/46 as a readout of mTORC1 activity (97,111,112).

1.17.2 mTORC2 complex

In addition to mTOR, this complex contains many other proteins such as Rictor, Protor, and mSIN1, which are not present in the mTORC1 complex. mLST8 and DEPTOR are found in both complexes.

Rictor (**R**apamycin **I**nsensitive **C**ompanion of **TOR)** is approximately 200 kDa protein, which competes with Raptor for mTOR binding. It is also considered to confer Rapamycin insensitivity to mTORC2 (113).

Protor-1 (**P**rotein observed with Rictor-1 also known as PRR5) is 42kDa protein that binds to Rictor. Protor exists in 2 isoforms, both of which can bind to Rictor. Protor is not required for mTORC2 complex stability or activity (114).

mSIN1 (mammalian **S**tress activated protein kinase **i**nteracting protein) is a 57 kDA protein that forms a complex with Rictor. mSIN1 is shown to modulate Rictor phosphorylation, which helps in stabilization of mTORC2 complex. mSIN1 is also important for mTORC2 activity (115).

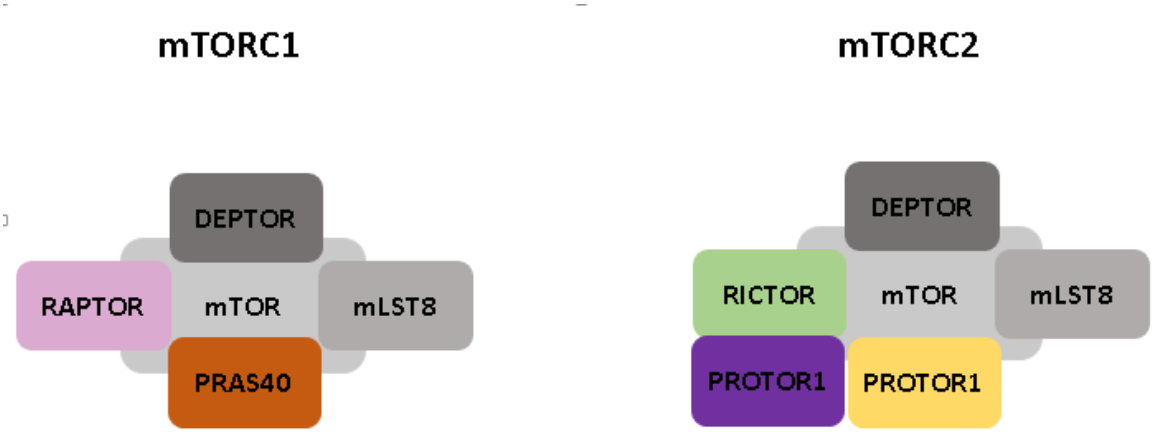


Figure 6 : Components of mTOR complex1 and mTOR complex 2

Both complexes have some common components, mTOR: mammalian target of Rapamycin, DEPTOR: DEP domain containing mTOR interacting protein, mLST8: Mammalian homolog of lethal with sec-13 gene 8.

mTORC1 complex components are Raptor (Regulatory associated protein of mTOR), PRAS-40 (Proline Rich AKT substrate 40 KDa)

mTORC2 complex components are Rictor (Rapamycin insensitive companion of TOR), Protor-1 (Protein observed with Rictor 1), mSIN1 (mammalian stress activated kinase interacting protein 1)

1.18 Regulation of mTORC1 pathway

mTORC1 acts as a central sensor for cells integrating various signals from growth factors, nutrient availability, energy status, glucose, oxygen and that regulates many physiological functions such as metabolism. It is an evolutionary conserved pathway that responds to nutrients**.** (101-103,116)

Most of the upstream signals regulate mTORC1 by two mechanisms

1. Direct modification of mTORC1 components
2. Regulation of RHEB.

Recently, the role of Rag–Ragulator complex in mTORC1 localization and activation has been demonstrated. Rags are small GTP-binding proteins that play an important role is activation of mTORC1 by amino acids. Recently, the newly discovered Rag GTPases (RagA-D) represent a key development in understanding of mTORC1 regulation by amino acids. They act as heterodimers either of Rag A or B (Rag A and B are redundant in function) with Rag C or D (Rag C and D are redundant in function) and reside on lysosomal membrane with the help of Ragulator (multimeric complex of MP1, p14, p18). In amino acid deprivation condition, RagA/B is bound to GDP and RagC /D to GTP (117-120).

Amino acids induce change in the nucleotide-bound status of Rag proteins by unknown mechanism resulting in RagA/B bound to GTP and RagC/D to GDP, indicating active state of heterodimer. The RagB-GTP/RagC-GDP heterodimer is shown to be the most effective one. The active heterodimer serves as a docking site on the lysosomal surface for mTORC1 through interaction with Raptor where

mTORC1 can interact with Rheb-GTP for its activation. As activation of mTORC1 by Rheb is also an upstream event for other signaling pathways such as by growth factors, localization of mTORC1 to lysosome by Rags might be the key event in mTORC1 signaling (118-121).

Current model of mTORC1 pathway

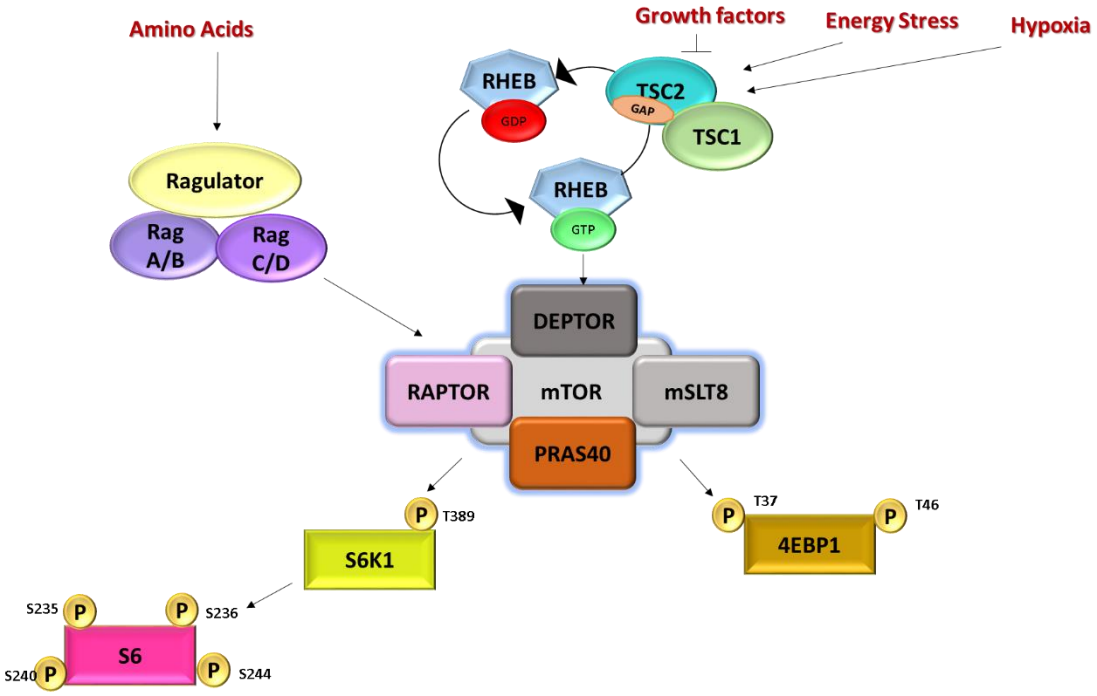


Figure 7 : Current model of mTORC1 signaling pathway

Various signals such as Amino acids, growth factors, energy stress, and hypoxia regulate the mTORC1 pathway. Amino acids regulate mTORC1 via the Ragulator-Rags complex by lysosomal localization. Rheb is a small GTPase that acts as a positive regulator of mTORC1 and is negatively regulated by the TSC1/TSC2 complex via GAP activity of TSC2 towards Rheb. Activity of the TSC1/TSC2 complex is regulated by phosphorylation of TSC2 on specific sites. Activated mTORC1 complex can phosphorylate its substrates such as S6K1 and 4EBP1. Phosphorylation of S6K1 at T389 and phosphorylation of 4EBP at T37 and T46 are used as a readout for mTORC1 activity. Phosphorylation of ribosomal protein

S6 at 4 sites (S235, S236, S240, and S244) by S6K1 is also used as a marker for mTORC1 activity.

1.19 Upstream Regulators of mTORC1

To understand mTORC1 signaling, its upstream regulators, effect of various signals and well known downstream effectors of mTORC1 involved in cellular functions will be discussed.

1.19.1 TSC2/TSC1 complex:

TheTSC2/TSC1 complex is a negative regulator of mTORC1 signaling.TSC2, also known as Tuberin is encoded by the *Tuberous Sclerosis Complex 2* gene and TSC1 also known as Hamartin, is encoded by the *Tuberous Sclerosis Complex 1* gene; both are tumor suppressor genes. Their mutations result in a genetic disorder known as Tuberous Sclerosis Complex characterized by benign tumors containing large cells in different tissues (123). TSC2 and TSC1 are phosphoproteins. Various kinases have been reported to act more on TSC2 compared with TSC1. TSC2 also shows GAP (GTPase activating protein) towards RHEB (positive regulator of mTORC1) When TSC2/TSC1 complex is active it stimulates a change of Rheb-GTP to Rheb-GDP and inhibits mTORC1 activity. TSC1 does not have GAP activity and it is considered to be important for stabilizing TSC2 by forming a complex (124-127). In response to growth factors, TSC2 is phosphorylated by AKT on at least 5 different sites (S939, S981, S1130, S1132, T1462) resulting in its repression. The effect of TSC2 phosphorylation on its GAP activity is not clear (128).

1.19.2 RHEB

Rheb (Ras Homolog Enriched in brain) is a small GTPase protein which acts as a positive regulator of mTORC1. It belongs to the RAS superfamily and is conserved in eukaryotes. Even though the name suggests brain, Rheb is ubiquitously expressed in all tissues. It has an intrinsic GTPase activity thereby hydrolyzing GTP to GDP. This activity is stimulated by the GAP activity of TSC2 (125,129). It has been shown that both GDP- and GTP- bound Rheb, and even nucleotide free Rheb, can bind to mTORC1, but only GTP-bound Rheb can activate mTORC1(121,130).

1.20 Upstream signals in regulation of mTORC1

1.20.1 Growth factors signaling

It is well known that growth factors such as insulin and IGF-1 can activate mTORC1 signaling via the PI3K-AKT pathway. When growth factors are available, mTORC1 promotes anabolic processes such as protein synthesis, lipid biosynthesis, and nutrient storage (101-103). In presence of growth factors, activated AKT can phosphorylate TSC2 on at least 5 sites (S939, S981, S1140, S1132, and Thr1462). S939 and T1462 are shown as specific AKT sites and are conserved among species but other sites responsible for regulation of TSC2/TSC1 complex might exist. How TSC2 phosphorylation affects TSC2/TSC1 complex and its activity towards Rheb is not clear. Moreover, it has been shown that phosphorylation of TSC2 doesn’t change its GAP activity towards Rheb. Another study suggested a possible alteration in TSC2 subcellular localization by

phosphorylation, so it cannot act as GAP for Rheb. TSC1 has a transmemebrane domain that mediates localization of the TSC1/TSC2 complex in the membrane region. Many AKT targets are regulated by creating binding sites for 14-3-3 proteins after phosphorylation. One study suggested that phosphorylation of TSC2 on both S939 and Thr1462 is required for binding with 14-3-3 proteins. This binding results in dissociation of the TSC1/TSC2 complex and translocation of phosphorylated TSC2 (S939/S981) at cytosol alone. This disrupts physical proximity of TSC2 and Rheb, so TSC2 can no longer act as Gap for Rheb at the membrane, thus resulting in activation of Rheb. (131,132). mTORC1 is also regulated by growth factors via TSC2 independent mechanisms. Growth factor-activated AKT phosphorylates PRAS40, resulting in inhibition of its repression on mTORC1 (133).

A recent study demonstrated that in response to growth factors, accumulation of Phosphatidic acid (PA) in phospholipase-D (PLD)-dependent manner, results in activation of mTORC1. It was also shown that PA binds to the FRB domain of mTOR. Other data suggest that PA activation of mTORC1 might be dependent on TSC2, as loss of TSC2 results in activation of PLD-1 (134,135).

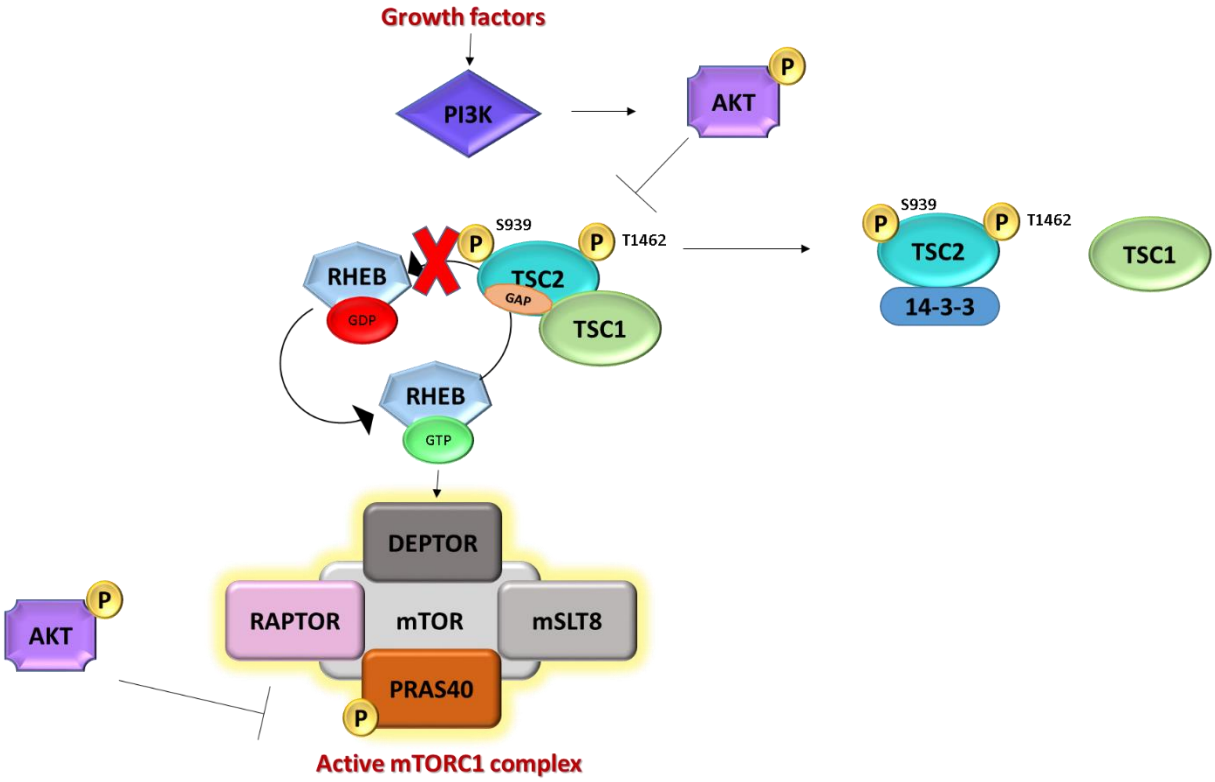


Figure 8 : Activation of mTORC1 complex by growth factors

Growth factors such as Insulin and Insulin like growth factors activate the PI3K-AKT pathway. Activated AKT phosphorylates TSC2 on two conserved sites S939 and Thr1462, resulting in inhibition of the TSC1/TSC2 complex. Inhibited complex can't act as GAP (GTPase activating protein) for Rheb resulting in Rheb-GTP. Rheb-GTP activates mTORC1. Activated AKT also phosphorylates PRAS40 which negatively regulates mTORC1. Phosphorylation of PRAS40 results in its dissociation from mTORC1 and activation of mTORC1.

1.20.2 Glucose and Energy

Along with mTORC1 signaling, anabolic processes require energy in the form of ATP. Glucose deprivation also leads to lower ATP levels. Low ATP results in a higher AMP/ATP ratio, resulting in binding of AMP to AMP activated protein kinase (AMPK) and triggering its phosphorylation by LKB1. Activated AMPK phosphorylates TSC2 on S1387 and T1271 resulting in activation of TSC1/TSC2 complex and inhibition of mTORC1 through unknown mechanism (136,137).

Another study demonstrated that phosphorylation of TSC2 on S1387 primes it for further phosphorylation on 4 sites by glycogen synthase kinase 3β (GSK3β), which is regulated by Wnt signaling. This results in activation of theTSC1/TSC2 complex and inhibition of mTORC1. Wnt signaling inhibits GSK3β and can activate mTORc1 signaling. However, the physiological condition leading to cross talk between energy sensing and Wnt signaling is not clear (138). mTORC1 can sense energy depletion independent of TSC2, partly by phosphorylation of Raptor on two conserved residues by activated AMPK. Phosphorylation of Raptor induces binding of 14-3-3 to raptor and inhibition of mTORC1 by a poorly understood mechanism (139).

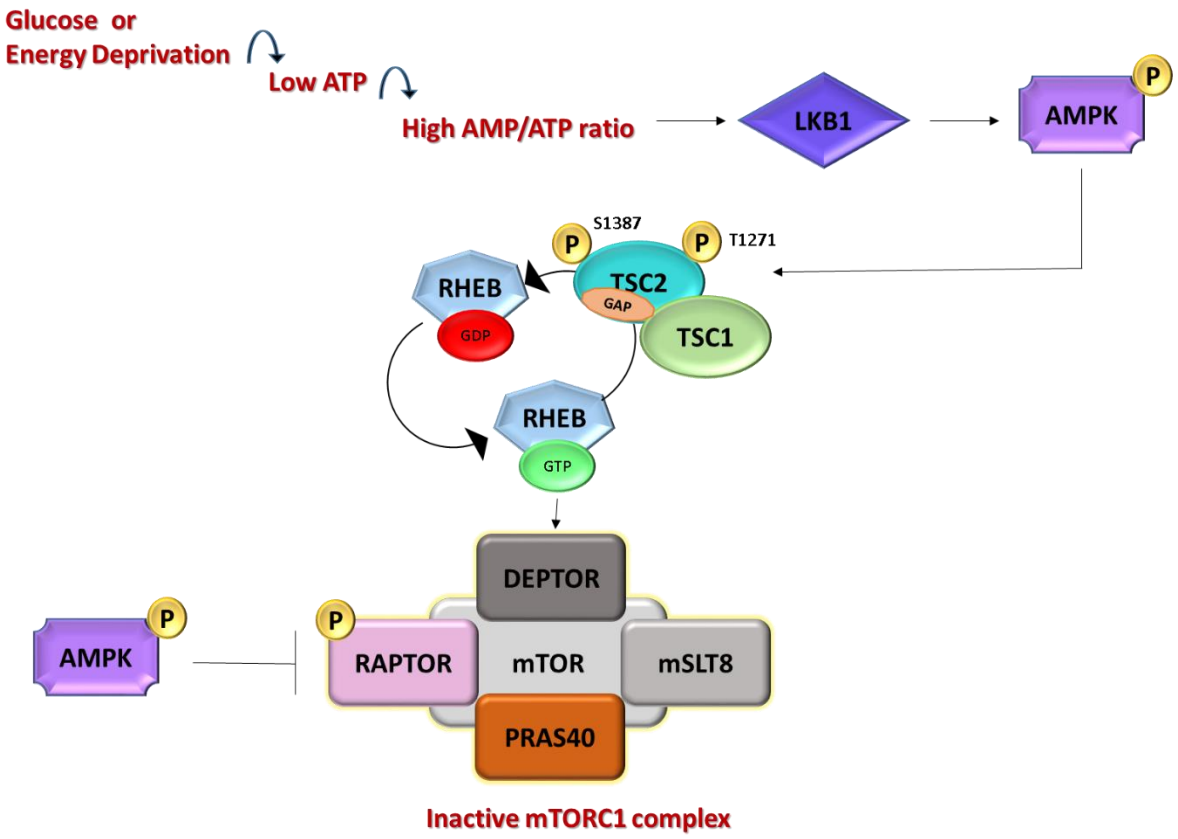


Figure 9 : Low Glucose and energy levels result in an inactive mTORC1 complex

Low glucose or energy result in lower levels of ATP and increased AMP/ATP ratio. This results in activation of the AMP kinase (AMPK) by LKB1. AMPK phosphorylates TSC2 on S1387 and T1271 resulting in activation of TSC1/TSC2 complex. This complex stimulates Rheb-GTP to Rheb-GDP, resulting in inactivation of mTORC1.

1.20.3 Hypoxia (Low Oxygen level)

mTORC1 is inhibited by low oxygen levels (hypoxia). Hypoxia results in inhibition of aerobic ATP production resulting in reduction of cellular ATP levels, resulting in activation of AMPK and inhibition of mTORC1, as described above.

Another mechanism involves REDD1, a cytoplasmic protein which is induced under hypoxic conditions (123). Recently it was demonstrated that REDD1 reverses AKT-mediated inhibition of TSC1/TSC2 complex. REDD1 activates the TSC1/TSC2 complex by moving 14-3-3 proteins bound on two AKT phosphorylation sites on TSC2 (S939, Thr1462) (140,141). Other pathways also have been proposed for suppression of mTORC1 by hypoxia, but more information is required.

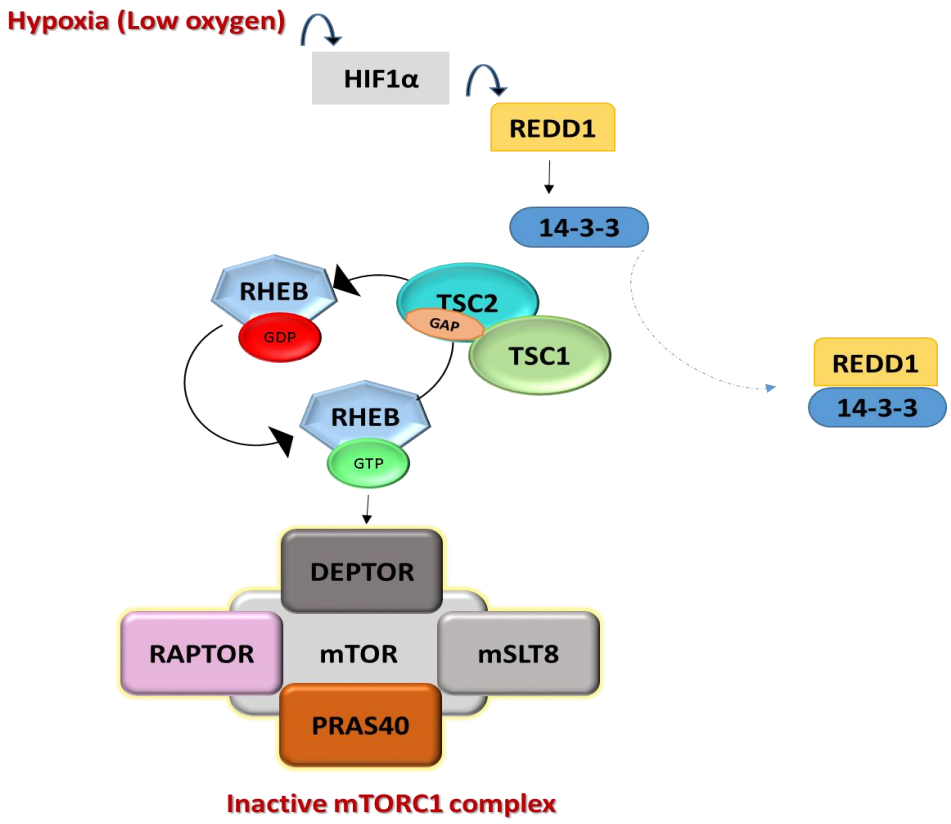


Figure 10 : Hypoxia results in inactivation of mTORC1

Hypoxia induces HIF1α transcription factor which induces REDD1. REDD1 moves 14-3-3 proteins away from TSC1/TSC2 complex resulting in activation of complex. This complex stimulates Rheb-GTP to Rheb-GDP resulting in inactivation of mTORC1.

CHAPTER II

MATERIALS AND METHODS

2.1 Animal Experiments

All animal experiments were conducted in accordance with regulations of IACUC at Cleveland State University. C57BI/6J mice were a gift from Dr. Bradfield and Dr. Marina Antoch. *Bmal1-/-* mice have been previously described. In all experiments male mice between ages of 3-6 months were used. Unless otherwise noted, all mice were maintained under standard animal housing conditions, with free access to food and water in 12 hours light/ 12 hours dark cycles. For time restricted feeding experiments, definite amount of food (95% of daily consumption) was provided at ZT14 (2hrs after lights were off). All mice were kept for 2 weeks with restricted feeding regimen before starting experiments. For all experiments, animals were euthanized using CO_2 chamber standard protocol dictated by

Cleveland State University. For starvation experiments, definite amount of food (95% of daily consumption) was provided at ZT14 and then no food was provided for next 48 hrs. Animals were sacrificed every 2 or 4 hr within this period.

2.2 Tissues, Cells and Cell culture

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % Fetal Bovine serum (FBS) and 10,000 units of Penicillin G and 10,000 µg/ml streptomycin. Cells were maintained at 37˚C at 5% CO_2 condition.

To generate primary lung fibroblasts, lung tissues isolated from WT and *Bmal1-/-*mice were cut into small pieces and plated in DMEM media. After 72 hr, media was replaced to remove unattached cells. Then cells were passaged every 3 days. During each passage, cells were counted and plated at equal density in each plate during every passage Cells were immortalized by passaging and then immortalized cell lines were used for experiments.

To collect tissues, after sacrificing animals, tissues were quickly frozen on dry ice and stored in -80˚C. For histological purpose, isolated tissues were immediately frozen in OST solution.

2.3 Senescence associated β-gal staining detection

Accumulation of senescent cells is used as cellular marker of aging. Senescent cells in isolated tissues were detected using senescence associated β-gal staining (SA-βGal) using a previously described protocol (56). Briefly, animals

were sacrificed and tissues were immediately frozen using OST solution. Four µm sections were fixed in 4% paraformaldehyde/PBS solution and stained using a x-Gal/potassium Ferro cyanide/potassium ferricyanide protocol. Stained tissue sections were analyzed under microscope and photographed. The number of blue cells was counted by 3 people independently blind to genotype. Sections were counterstained with hematoxylin /eosin according to standard protocol.

2.4 Replicative senescence

Replicative senescence represents primary cell growth arrest in cell culture. For measuring replicative senescence, primary lung fibroblasts from WT and *Bmal1-/-*mice were isolated as mentioned before. Cells were split as per standard 3T3 protocol. Briefly cells were passaged every 3 days and during each passage, cells were counted using hem cytometer and all cells were plated again at density of $10^4/cm^2$. The procedure was followed until the time, the cells became immortalized. (n=16 8WT 8 KO). Cell number at each passage was used as measure of cell proliferation. Reduction in number of cells after few passages was used as an indication of senescence and was confirmed using SA-βgal staining.

2.5 Oxidative stress sensitivity assay

For the assay, WT and *Bmal1-/-* cells were plated at 1000 per well in three 96-well culture dishes. At 24 hr. after plating, cells were treated with different concentrations of Hydrogen peroxide (Sigma Aldrich H1009-500ML) for 24, 48 and 72 hr. Treated cells were washed with PBS and fixed using 0.2% Glutaraldehyde prepared from 25% Glutaraldehyde (Fisher Scientific) by diluting in PBS. Fixed

cells were stained using 0.05% crystal violet solution for 30 min on shaker. Dye was extracted using 1% SDS (Invitrogen) solution and amount of dye incorporated was measured as optical density at 570 nM using a Victor 1420 plate reader.

2.6 Colony forming assay

To check effect of oxidative stress on cell proliferation, we performed colony forming assay. WT and *Bmal1-/-* immortalized lung fibroblasts were plated at a density of 10000 per well on 6-well culture dishes. Next day after plating, one well of each WT and *Bmal1-/-* was either treated with DMEM (untreated) for 96 hr, or in presence of 250 nM Hydrogen Peroxide for 96 hr, or in presence of 250 nM Hydrogen Peroxide for 24 h. and then regular media for next 72 hr. After 96 hr of treatment, cells were washed with PBS, fixed using 0.2% Glutaraldehyde and stained using 0.05% Crystal Violet solution for 30 min on shaker. Dye was removed, plated cells were washed gently with water to remove excess dye and air dried. Stained colonies were counted by 3 people independently blind to cell genotype.

2.7 Cell death assay

Damaged cell membrane and leakage of intracellular contents in media are indications of cell death. In this assay, membrane damage was detected using the CytoTOX-ONE^{TM} Homogenous Membrane integrity assay (Promega). Briefly, WT and *Bmal1-/-* immortalized lung fibroblasts were plated at a 1000 density in 96-well culture plates. After 24 hr following plating, fibroblasts were either treated with DMEM, or with different concentrations of Hydrogen Peroxide for 4Hrs. in the 37˚C

incubator. After 4Hr of incubation, positive control wells were treated with Lysis solution reagent provided by manufacturer as a positive control for cell death. Equal volume of CytoTOX-ONE^{TM} was added in each well and mixed on shaker for 30 sec. Plates were incubated at room temperature for 10 min and fluorescence was recorded using Victor 1420 plate reader. The fluorescence was recorded every 10mins for 1 hr.

2.8 Anticancer Drugs sensitivity assay

To examine changes in genotoxic stress resistance of *Bmal1-/-* fibroblasts, we used different anticancer drugs. These drugs were chosen in such a way that they act through different pathways and affect cell proliferation or cell death through different mechanisms. Following drugs were used,

- Paclitaxel (Taxol): Microtubule stabilizing agent
- 5-Fluorouracil: Antimetabolite
- Etoposide: DNA damaging by producing double-stranded breaks
- Daunorubicin: Antimitotic activity

For the assay, WT and *Bmal1-/-* immortalized lung fibroblasts were plated at 1000 per well in three 96-well culture dishes. Twenty four hours after plating, cells were treated with different concentrations of either Paclitaxel, 5-Flurouracil, Etoposide or Daunorubicin (Sigma-Aldrich) for 24, 48 and 72 hrs. Cell survival was estimated using two independent protocols, the MTT assay and Crystal Violet staining. The MTT assay was performed using In Vitro Toxicology assay kit (Sigma M6555) as per manufacturer's protocol. Briefly, the MTT reagent was reconstituted using

media just before use. Reconstituted reagent was added to treated plates in equal amount (10% of each culture volume).These cells were incubated for 2 hr and absorbance was measured at 570 nM using a Victor 1420 plate reader.

In crystal Violet staining, treated cells were washed with PBS twice and fixed using 0.2% Glutaraldehyde prepared from 25% Glutaraldehyde (Fischer Scientific). Fixed cells were stained using 0.05% crystal violet solution for 30 minutes on shaker. The dye was removed and excess dye was washed out by water. Incorporated dye was extracted by adding 1% SDS solution and shaking for 30 min on shaker. The incorporated dye was measured as optical density at 570 nM using Victor 1420 plate reader.

2.9 Rapamycin Sensitivity assay

Rapamycin (Sigma–Aldrich) was used as mTOR inhibitor. WT and *Bmal1-/-*immortalized lung fibroblasts were plated at equal density in three 96 well plates. One plate was fixed after 6 hr of plating as control. For fixing, media was removed and cells were washed with PBS twice and fixed with 0.2% glutaraldehyde. Two 96 well plates were treated with different concentrations of Rapamycin for 24 and 48 hr. Treated cells were stained and the dye was extracted as indicated in section 2.5. Optical density reading was used as indication of cell proliferation (the high optical density represents more cell proliferation).

2.10 Protein content determination

Plated WT and *Bmal1-/-* immortalized lung fibroblasts were trypsinized and collected in DMEM media. Cells were counted and an equal number of WT and

Bmal1-/- cells were collected in micro centrifuge tubes. We prepared different sets of WT and *Bmal1-/-* samples with different number of cells. Collected cells were washed twice in PBS and lysed using equal volume of RIPA buffer. Protein concentrations of cell lysates were measured using protein assay reagent (BIO-RAD 500-0006) using spectrophotometer. 1mg/ml albumin solution was used to make standard curve.

2.11 Western blotting analysis

2.11.1 Cell Lysates

WT and *Bmal1-/-* immortalized lung fibroblasts were plated at a density of 400,000 in 100 mm culture dishes. After 24 hr of plating, cells were treated as described in each experiment. Cells were washed twice with PBS and collected in 1ml PBS per tube. Tubes were centrifuged at 6000 rpm for 10 minat 4^0 C using Eppendorf 5417R centrifuge. Supernatant was discarded and pellet was re-suspended in 50-100 µl RIPA lysis buffer (Tris Base pH 7.4 10mM, NaCl ,EDTA pH8, NaF, 20 % SDS,Triton-X100,glycerol water). Just before use, Protease inhibitor cocktail (Sigma), Phosphatase inhibitor Cocktail2 (Sigma-Aldrich) at a concentration of 10 µl/ ml of buffer were added. Cell pellets were lysed by vortexing for 4-5 min. Lysates were centrifuged for 12 min at 10000rpm, 4^0C. Supernatants were used for protein concentration determination and lysates were stored at -80^0 C.

2.11.2 Tissue Lysates

Different tissues from WT and *Bmal1-/-* mice were collected over 24 period with interval of 4 hr. Tissues were quick frozen on dry ice and stored at -80^{0}C. For tissues lysates, a small piece was transferred into micro centrifuge tubes with 300 µl Cell Signaling Buffer (Tris pH 7.5, NaCl, 0.5M EGTA, 0.5 M EDTA, Triton-x 100, $Na_4P_2O_7$, β-glycerophosphate, 1M Na_3VO_4). Just before use, Protease inhibitor (Sigma), Phosphatase inhibitor Cocktail2 (Sigma-Aldrich) at a concentration of 10 µl/ ml of buffer were added. Tissues were lysed using a sonicator (Fischer Scientific Model 100) for 5-10 seconds on ice. Lysates were centrifuged for 12 min at 10000 rpm at4^{0}C. Supernatants were used for protein concentration determination and lysates were stored at -80^{0}C.

Protein concentrations of lysates were measured using the protein assay reagent (BIO-RAD 500-0006) as per manufacturer's instructions. Following protein concentration determinations, lysates were denatured using 2x Loading mix (300 mM Tris HCl PH6.8, 10% SDS, 50% Glycerol, 10% 2-mercaptoethanol, 0.0025% Bromophenol blue) and cell signaling buffer to adjust equal concentration of protein in all lysates. Proteins were separated using 4-12% NuPAGE Bis-Tris precast gels, starting at 90V and then at 145V (SDS-PAGE). Samples were transferred electrophoretically at 110 mA for 70 min on Polyvinylidene Difluoride membrane (Thermo Scientific 88518) using transfer buffer containing 20% methanol, Tris Base 3g/L, Glycine 14.4g/L. After transfer, membranes were placed in blocking solution of 5% non-fat dry milk in TBS-T buffer (Tris Base 60.57 g/L, NaCl 87.66 g/L PH adjusted to 7.4 with HCl and 0.1% Tween-20) for 1 hour at room temperature on shaker. Blots were incubated with specific primary and secondary antibodies as described in the table below **(Table1).** Western blotting analysis was performed using Clarity ^{TM} Western ECL Substrate (BIO-RAD) as instructed by the manufacturer and bands were visualized using Scientific Imaging film (Denville) and Odyssey FC imaging system (LI-COR). Bands were quantified using LI-COR software.

Table 1: Antibodies for Western Blotting

Antibody	Phosphorylation Site	Dilution	Washings	Catalogue	Company
P-mTOR	S2448	1:1000 in 5% BSA at 4°overnight	3 washings 5 minutes each	2971	Cell Signaling
P-mTOR	S2481	1:1000 in 5% BSA at 4°overnight	3 washings 5 minutes each	2974	Cell Signaling
mTOR	Total	1:1000 in 5% BSA at 4° overnight	3 washings 5 minutes each	2972	Cell Signaling
P-TSC2	T1462	1:1000 in 5% BSA at 4°overnight	3 washings 5 minutes each	3614	Cell Signaling
TSC2	Total	1:1000 in 5% BSA at 4°overnight	3 washings 5 minutes each	3990	Cell Signaling
P-S6K1	T421/S424	1:1000 in 5% BSA at 4°overnight	3 washings 5 minutes each	9204	Cell Signaling
P-S6K1	T389	1:3000 in 5% Milk at 4°overnight	3 washings 5 minutes each	9206	Cell Signaling
S6K1	Total	1:1000 in 5% BSA at 4°overnight	3 washings 5 minutes each	2708	Cell Signaling
P-S6	235/S236	1:5000 in 5% BSA at 4°overnight	3 washings 5 minutes each	2211	Cell Signaling
P-S6	S240/S244	1:5000 in 5% BSA at 4°overnight	3 washings 5 minutes each	2215	Cell Signaling

S6	Total	1:5000 in 5% Milk at 4°overnight	3 washings 5 minutes each	SC-74459	Santa Cruz
P-4EBP1	T37/46	1:1000 in 5% BSA at 4°overnight	3 washings 5 minutes each	2855	Cell Signaling
4EBP1	Total	1:1000 in 5% BSA at 4°overnight	3 washings 5 minutes each	9452	Cell Signaling
Deptor	Total	1:1000 in 1% BSA + 1% milk at 4°overnight	3 washings 5 minutes each	NBP1-49674	Novus biologicals
Rheb	Total	1:1000 in 1% BSA + 1% milk at 4°overnight	3 washings 5 minutes each	4935	Cell Signaling
B-Actin	Total	1:30000 in 5% BSA 1 hour Room temp.	3 washings 5 minutes each	A5441	Sigma-Aldrich
GAPDH	Total	1:30000 in 5% BSA 1 hour Room temp.	3 washings 5 minutes each	5174	Cell Signaling

2.12 Semi Quantitative RT-PCR

Total RNA was extracted from frozen tissue, using Trizol (Invitrogen) reagent as per manufacturer's instructions and some modifications. Briefly, for every piece of tissue 1 ml of Trizol reagent was used. Tissues were homogenized with pestle (Sigma-Aldrich) for at least 5 minutes or until the entire tissue was homogenized. This was done keeping tubes on ice. Tubes were spun at 4^0C at 11500 rpm for 10 min. Supernatants were transferred into new tubes and

incubated at room temperature for 5 minutes. After adding 200 µl chloroform (Fischer scientific), tubes were shaken vigorously and incubated for 2-3 minutes

at room temperature. After spinning for 15 minutes at 11500 rpm at 4^0C, the aqueous phase was transferred into a new tube. The RNA was precipitated by adding 500 µl isopropanol. RNA was washed using 70% ethanol and pellet was air dried for 5 minutes. The pellet was re-suspended in 30 µl of RNAse free water. The RNA concentration was determined using Nano drop. Following quantification, a 20µl reverse transcription reaction contained 1µg of RNA, superscript III (Invitrogen),DNTPs, Random hexamers, DTT and 5X manufacturer's buffer as per manufacturer's instructions . Real time PCR reaction was performed using forward and reverse primers (IDT), 0.5 µl cDNA and SYBRGreen PCR Super mix (BIO-RAD).Every reaction was performed in triplicates, amplified and quantified using Opticon 2 quantitative real time PCR system (MJ research).The Relative mRNA abundance was calculated using the comparative delta-Ct method with 18S RNA as standard.

Table 2: Primers for PCR

18s rRNA	Forward	GCT TM TU GAC TCA ACA CGG GA
	Reverse	AGC TAT CM TCT GTC AAT GTC
mtor	Forward	ATT CAA TCC ATA GCC CCG TC
	Reverse	TGC ATC ACT CGT TCA TCC TG
deptor	Forward	GTG GTT CTC AGG CAT TCT ATC TC
	Reverse	TGG GTA GGT TTT GAG ATG GTG

CHAPTER III

RESULTS

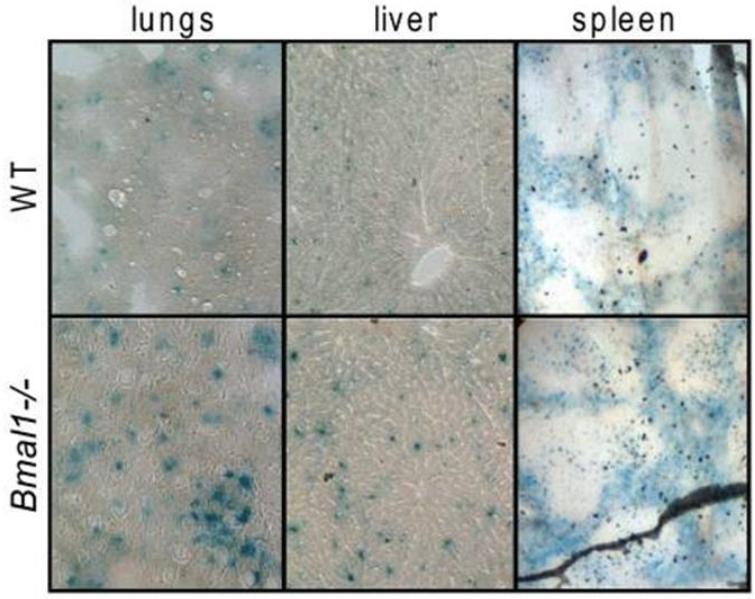
3. A. The circadian clock protein BMAL1 regulates cellular senescence *in vivo.*

3. A.1 *Bmal1* deficiency results in increased number of senescent cells *in vivo.*

Previously, our lab has shown that BMAL1 deficiency results in reduced lifespan and many age-related pathological conditions such as Sarcopenia (reduced skeletal mass), cataract, reduced memory, calcification and ossification of joints, thus linking circadian clock with aging. Other labs have shown that senescent cells accumulate with aging *in vivo*. Senescent cells are characterized by arrested cell proliferation, enlarged morphology, resistance to apoptosis and change in expression of proteins known as markers of senescence. These findings lead us to hypothesize that the circadian clock protein BMAL1 may

play a critical role in cellular senescence, which contributes to premature aging in *Bmal1-/-* mice. To test this hypothesis, we used different tissues (liver, lung, spleen) collected from 6-10 months old WT and *Bmal1-/-* mice. At this age, *Bmal1-/-* mice show multiple features of premature aging. Tissues were processed and stained for Senescence associated β-galactosidase staining (SA-β-gal). We observed that the intensity of blue staining developed in *Bmal1-/-* mice tissues was higher compared with WT tissues of the same age used as control, indicating a high SA-β-gal activity in *Bmal1-/-* mice **(Figure11A** *Khapre R V et al. Cell cycle. 2011 Dec 1; 10 (23):4162-9.*We counted the number of blue cells (SA-β-gal positive) in stained liver and lung tissues under the microscope and plotted as graph **(Figure 11B).**The difference in number of SA-β-gal positive cells was about 3 fold higher in lungs and liver but no clear difference obvious in the spleen. This indicates that accumulation of senescent cells is tissue-specific. We also stained lung tissues obtained from 1 month old WT and *Bmal1-/-* mice and found that even at this age when *Bmal1-/-* mice do not show any signs of premature aging, SA-β-gal activity was higher (Data not shown). These results supported our hypothesis that *Bmal1-/-* deficiency results in the accumulation of senescent cells in different tissues correlating with premature aging in *Bmal1-/-* mice.

A. Figure (A)



B. Figure (B)

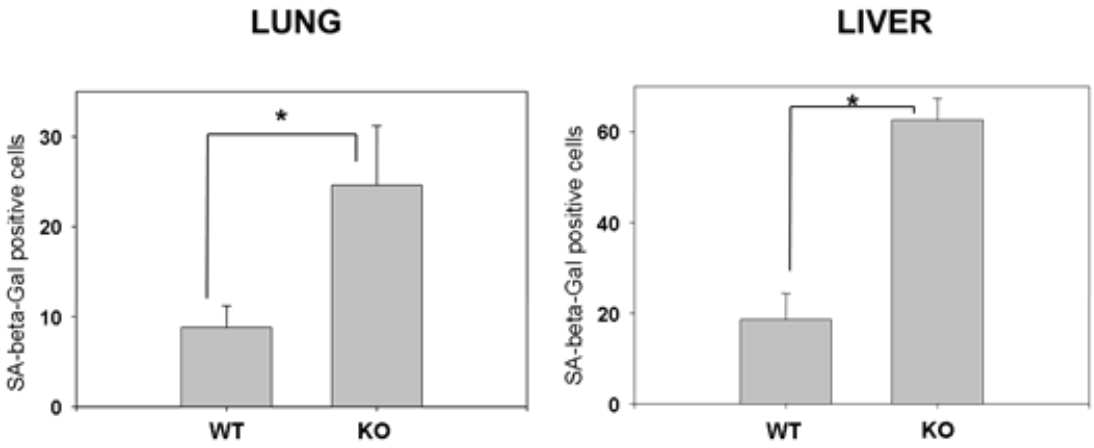


Figure 11 : *Bmal1* deficiency leads to accumulation of senescent cells *in vivo.*

Representative histological images of lung (left panel), Liver (middle panel), spleen (right panel) isolated from 6 months old WT mice (upper row) and *Bmal1-/-* (lower row) stained for SA-β-gal staining. **(A)** Collected tissues were stained and images were captured**.**

(B) Number of blue stained cells were counted by three individuals blind to the genotype and results are plotted as mean ± std. deviation (n= 10. 5WT 5KO).In case of lungs mean of SA-β-gal positive cells in WT is 8.8 ± 2.4 whereas in *Bmal1-/-* is 24.6 ± 6.6. In liver mean of SA-β-gal positive cells in WT is 18.6 ± 7.8 whereas in *Bmal1-/-* is 62.6 ± 4.7. (*) indicates $p < 0.05$

3. A.2 *Bmal1* deficiency does not affect cellular replicative senescence *in vitro*

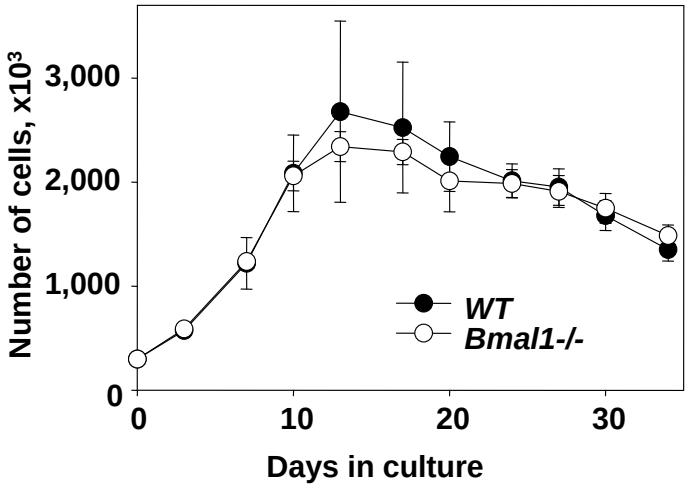
Once we observed the increased accumulation of senescent cells *in vivo* in Bmal1-/- *mice*, next we wanted to determine if *Bmal1* deficiency affects senescence *in vitro*. When primary cells are cultured *in vitro*, they demonstrate limited life span and undergo replicative senescence after dividing for definite number of times. We hypothesized that if tissues from *Bmal1-/-* mice show higher accumulation of senescent cells then primary cells isolated from *Bmal1-/-* tissues will undergo replicative senescence earlier compare with WT cells in culture. For this purpose, we decided to use primary lung fibroblasts from WT and *Bmal1-/-* mice for two reasons: 1) we had observed increased accumulation of senescent cells in *Bmal1-/-* lung tissues 2) Fibroblast are a well-known model for studying senescence.

For isolating lung fibroblasts, we cut lung into very small pieces and cultured them in DMEM. Everyday culture plates were examined for isolation of fibroblasts from tissue pieces. In 3-4 days, when some fibroblasts were attached, half volume of media (DMEM) was replaced with new media. This was done to provide more nutrients but at the same time, to retain growth factors secreted by fibroblasts in culture. After 5 days of isolation, fibroblasts were split for the first time as first passage. After the first passage, cells were passaged every 3 days following NIH 3T3 protocol. During every passage, number of cells was counted using hemocytometer and all the cells were plated again at equal density. The procedure

was repeated until cells were immortalized. As expected, most of the fibroblasts from WT and *Bmal1*-/- mice did undergo senescence. But when we compared proliferation curves and the onset of replicative senescence between WT and *Bmal1-/-*fibroblasts, we observed no significant difference (Figure 12 A from *Khapre R V et al. Cell cycle. 2011 Dec 1; 10 (23):4162-9*). This indicated that *Bmal1* deficiency might not significantly affect cellular replicative senescence *in vitro* and most likely does not contribute to increased cellular senescence *in vivo* in *Bmal1-/-* mice.

It is known that mouse primary fibroblasts can spontaneously immortalize in culture. As expected, our WT and *Bmal1-/-* primary lung fibroblasts did undergo immortalization after several rounds of passaging. Unexpectedly, we observed that *Bmal1-/-* primary lung fibroblasts showed a tendency to immortalize at earlier passages compare to WT (average number of passages for before immortalization for wild type 14.5$\pm$ 1.5 compared to 11.4 $\pm$ 1.5 for *Bmal1-/-*) **(Figure 12B)**.Thus, unlike other models of aging, *Bmal1* deficiency does not result in premature replicative senescence of primary fibroblasts in culture and different mechanisms might be responsible for accumulation of senescent cells in *Bmal1-/-* organs.

A.



B.

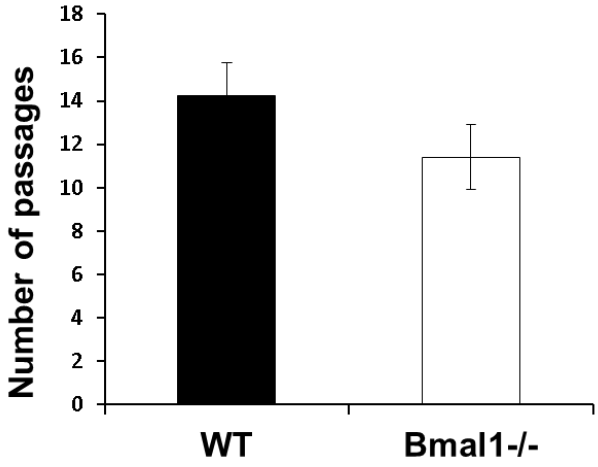


Figure 12 : *Bmal1* deficiency does not affect replicative senescence *in vitro*.

Lungs tissues were cut and placed in media to isolate primary fibroblasts. During every passage cells were counted and plated at equal density on the indicated days. (A) Cellular proliferation curves of primary lung fibroblasts for WT (indicated by closed circles) and *Bmal1-/-* (indicated by open circles WT and *Bmal1-/-*

proliferation curves show no difference in the onset of replicative senescence, as observed at later passages). Results are plotted as mean ± std. deviation (n= 16 8WT, 8KO).

(B) Immortalization of primary lung fibroblasts from WT and *Bmal1-/-* mice. As indicated in the figure, *Bmal1-/-* primary lung fibroblasts showed tendency to immortalize at earlier passages compare to WT (average number of passages for before immortalization for wild type 14.5± 1.5 and 11.4 ± 1.5 for *Bmal1-/- cells*). Results are plotted as mean ± std. deviation (n= 16 8WT, 8KO).

3. A.3 *Bmal1* deficiency results in increased sensitivity to oxidative stress *in vitro*

The role of replicative senescence in accumulation of senescent cells *in vivo* is unclear. Stress-induced senescence might be a contributor. It can be induced by challenging cells with different stimuli such as DNA damage, Oncogene activation or aberrant mitogenic signals. Oxidative stress has been suggested as major contributor to stress-induced senescence. Previously, our lab has shown that BMAL1 is involved in Reactive oxygen species (ROS) homeostasis and life-long treatment with antioxidant significantly increased the average and maximal life span of *Bmal1-/-* mice. Based on these data, next we hypothesized that BMAL1 deficiency would result in increased sensitivity to oxidative stress. To test this hypothesis, we used hydrogen peroxide as an oxidative stress inducer. Immortalized WT and *Bmal1-/-* lung fibroblasts were plated at equal density in three 96 well plates. After 24 hours of plating, cells were treated with regular media or media with different concentrations of hydrogen peroxide for 24, 48 and 72 hours (**Figure 13** from *Khapre R V et al. Cell cycle. 2011 Dec 1; 10 (23):4162-9*).

As expected, *Bmal1-/-* fibroblasts showed increased sensitivity to hydrogen peroxide indicated by a decreased number of cells after treatment, as detected using crystal violet. These results supported our hypothesis that BMAL1 is involved in the oxidative stress response.

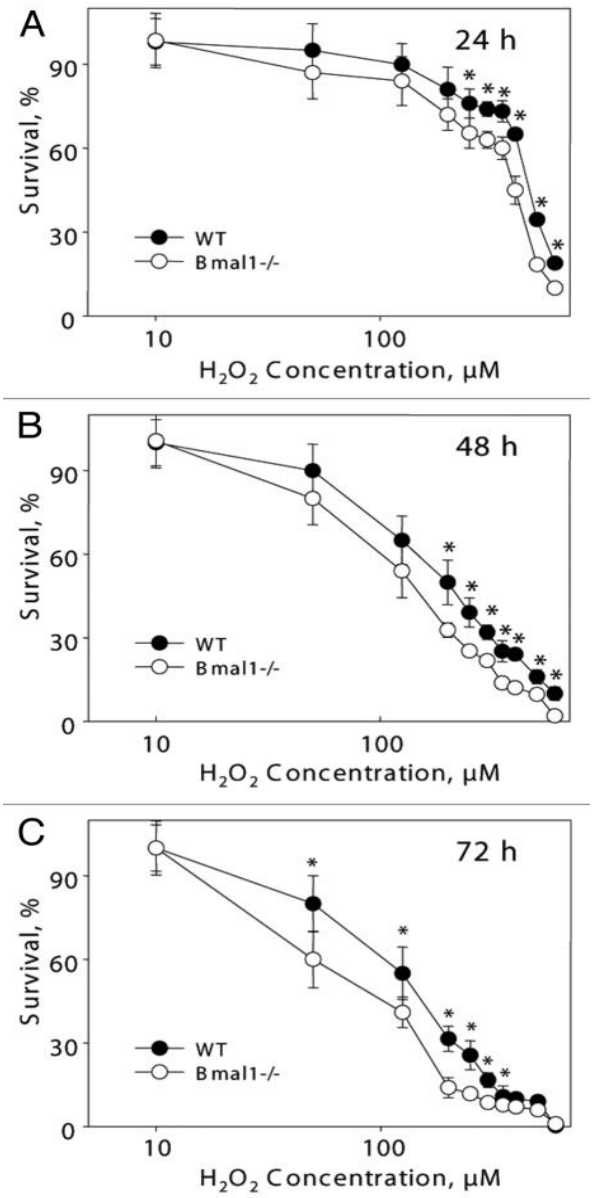


Figure 13 : *Bmal1* deficiency results in increased sensitivity to oxidative stress

Cells were plated at an equal density and treated with different concentrations of hydrogen peroxide for A) 24 B) 48 and c) 72 hours. The figure above shows survival curves for WT (closed circles) and *Bmal1-/-* (open Circles) fibroblasts at different concentrations of hydrogen peroxide. Survival of untreated cells were set

to 100%. *Bmal1-/-* cells were significantly more sensitive than WT cells. (*) $P < 0.05$. Results are plotted as mean ± std. deviation (n= 5).

24 hours WT IC50 = 450 µM *Bmal1-/-* IC50 = 385 µM (A)

48 hours WT IC50 = 200 µM *Bmal1-/-* IC50 = 140 µM (B)

72 hours WT IC50 = 150 µM *Bmal1-/-* IC50 = 87.5 µM (C)

3. A.4 *Bmal1* deficient cells are more sensitive to oxidative stress-induced growth arrest

After determining that *Bmal1-/-* cells are more sensitive to oxidative stress, indicated by a decreased number of cells present after hydrogen peroxide treatment, next we wanted to investigate the effect of oxidative stress on *Bmal1-/-* cells. Oxidative stress can either induce cell arrest or cell death, so the next step was to investigate if the increased sensitivity of *Bmal1-/-* cells was due to increased cell death or reduced proliferation. For both experiments WT and *Bmal1-/-* immortalized lung fibroblasts were used. To examine the effect of oxidative stress on cell death, we used CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega) as per manufacturer's instructions. Cells were plated at an equal density in 96-well plates and treated with different concentrations of hydrogen peroxide. At different time points, cells were fixed and treated with cell death kit reagents for assay. Fluorescence was measured using a Victor Wallac counter.

To examine the effect of oxidative stress on cell proliferation, we performed colony forming assay, following a concentration of hydrogen peroxide at which the difference in sensitivity to oxidative stress was maximum. Cells were plated at an equal density and treated with either regular media or 250 µM hydrogen peroxide for 96 hours or treated with hydrogen peroxide for 24 hours and then in regular media for an additional 72 hours. Cells were fixed and stained with crystal violet and formed colonies were counted.

After hydrogen peroxide treatment, we did not observe any significant difference in the rate of cell death between WT and *Bmal1-/-* cells (**Figure 4A** Figure from *Khapre R V et al. Cell cycle. 2011 Dec 1; 10 (23):4162-9.)*

In a cell proliferation assay, there was no difference in the number of colonies for untreated cells. Hydrogen peroxide treatment resulted in reduced number of colonies in WT and *Bmal1-/-* cells but the effect was significantly stronger in *Bmal1-/-* cells (**Figure 4B** Figure from *Khapre R V et al. Cell cycle. 2011 Dec 1; 10 (23):4162-9).* We also observed that reduction in number of colonies after hydrogen peroxide treatment was not as dramatic as reduction in colony size This indicated that oxidative stress induced growth arrest was stronger in *Bmal1-/-* cells. This was also supported by another observation that when cells were treated for 24 hours with hydrogen peroxide and then allowed to grow for additional 72 hours in regular media, no difference between genotypes was observed (**Figure 14C** Figure from *Khapre R V et al. Cell cycle. 2011 Dec 1; 10 (23):4162-9)*.

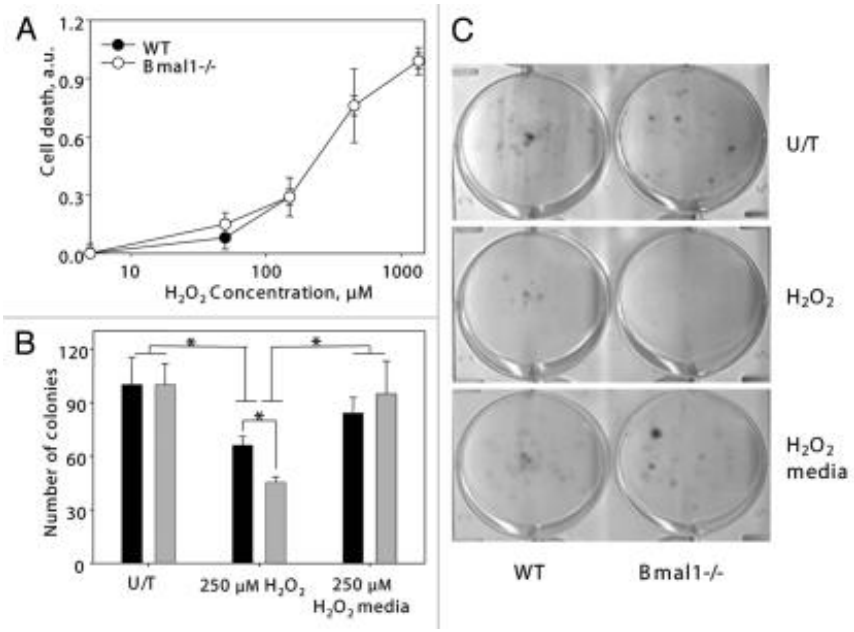


Figure 14 : *Bmal1 deficient* cells are more sensitive to oxidative stress induced growth arrest.

(A) Cell death curves for WT (closed circles) and Bmal1-/- (open circles) fibroblasts plated at an equal density and after treatment with different concentrations of hydrogen peroxide. With increase in hydrogen peroxide concentration, there was increase in cell death but no difference was observed between WT and Bmal1-/- cells. (B) The number of colonies formed by WT (black bars) and Bmal1-/- gray bars) immortalized lung fibroblasts. Cells were plated at equal density and treated with regular media (U/T) for 96 hours or in presence of 250 µM of hydrogen peroxide (H2O2) or treated with 250 µM hydrogen peroxide for 24 hours and then in regular media for additional 72 hours (250 µM H2O2/media.(C) Representative Images of Colony Forming assay. Colonies were stained with crystal violet and

excess dye was washed out with water. These curves are average of 4 independent experiments plotted as mean + std. deviation.

3. A.5 Differential effect of *Bmal1* deficiency on cell sensitivity to anti-cancer drugs.

After determining that *Bmal1-/-* cells are more sensitive to hydrogen peroxide treatment, next we wanted to investigate if the observed increased sensitivity of *Bmal1-/-* cells to hydrogen peroxide treatment is specific to oxidative stress or is a consequence of changes in genotoxic stress resistance. To explore the role of *Bmal1* deficiency in general genotoxic stress, we compared resistance of WT and *Bmal1-/-* lung immortalized fibroblasts to different anticancer drugs. These drugs were selected in such a way that they act through different pathways and affect cell proliferation, cell death through different mechanisms. Assays were performed in similar way as mentioned for hydrogen peroxide.

The different anticancer drugs showed a different effect on *Bmal1-/-* cells. Paclitaxel (Taxol) is a microtubule stabilizing drug. Microtubules are tubular polymers of α and β tubulin dimers. Paclitaxel binds to β-tubulin polymer and stabilizes microtubules by disturbing the steady state equilibrium between tubulin dimers and microtubules. These result in changes in dynamicity of microtubules, mitotic cell arrest, and cell death. Treatment with paclitaxel did not show any significant difference in sensitivity of WT and *Bmal1-/-* cells **(Figure 15A** from *Khapre R V et al. Cell cycle. 2011 Dec 1; 10 (23):4162-9*). Next we tested 5-fluorouracil which is an antimetabolite, a pyrimidine analogue that causes scarcity of thymine leading to cell death. Treatment with 5-fluorouracil also did not show any significant difference in the sensitivity of WT and *Bmal1-/-* cells **(Figure 15B** from *Khapre R V et al. Cell cycle. 2011 Dec 1; 10 (23):4162-9*). Treatment with Daunorubicin

(antimitotic activity) also showed similar results. *Bmal1-/-* cells were more resistant to Daunorubicin than WT (WT IC50 = 45.35 nM, *Bmal1-/-*, IC50 = 95.5 nM). (*) *P < 0.05* (**Figure 15C** from *Khapre R V et al. Cell cycle. 2011 Dec 1; 10 (23):4162-9*). We also tested Etoposide (topoisomerase II inhibitor). This assay showed that *Bmal1-/-* cells were more resistant to Etoposide compare with WT. (WT IC50 = 56.33 ng/ml, *Bmal1-/-* IC50 = 110.9 ng/ml) (*) *P < 0.05* **(Figure 15D** from *Khapre R V et al. Cell cycle. 2011 Dec 1; 10 (23):4162-9*). These results indicated that *Bmal1* deficiency does not change sensitivity to all drugs. *Bmal1-/-* deficiency did not affect cell sensitivity to Paclitaxel and 5-fluorouracil but made cells more resistant to other drugs such as Etoposide and Daunorubicin.

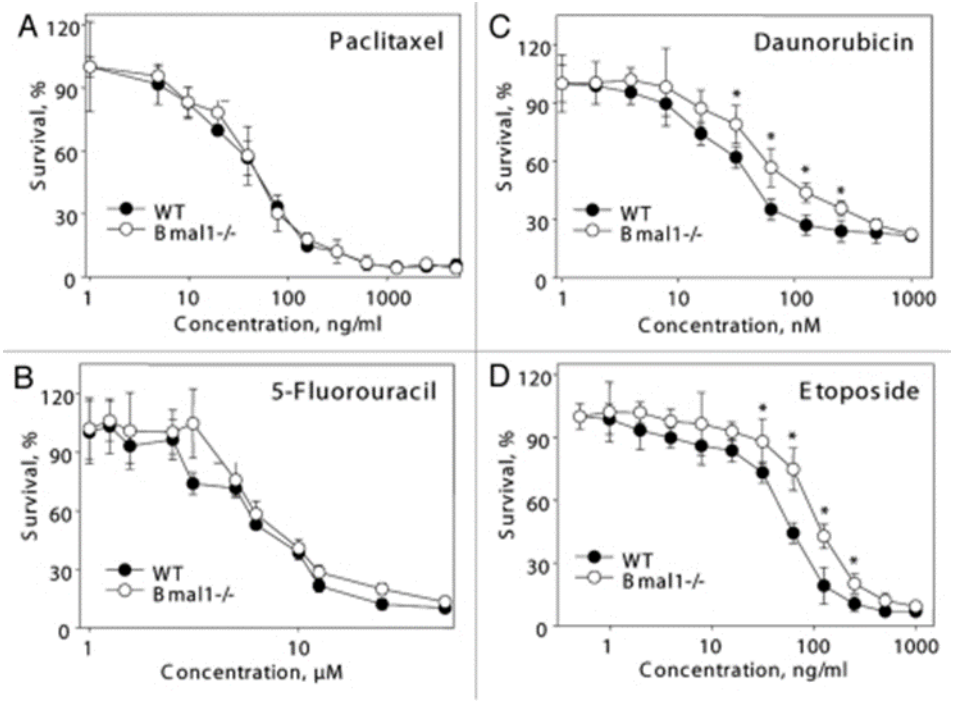


Figure 15 : *Bmal1* deficient cells have different sensitivity to anticancer drugs.

Relative survival curves of WT and *Bmal1-/-* immortalized lung fibroblasts plated at equal densities and treated with different concentrations of (A) Paclitaxel, (B) Daunorubicin, (C) 5-fluorouracil, (D) Etoposide. Survival of untreated cells was set to 100%.The curves are average of 3 independent experiments. As shown in (A) and (B), *Bmal1-/-* cells did not show any significant difference in sensitivity to paclitaxel and 5-Flurouracil when compared with WT. But, *Bmal1-/-* cells showed higher resistance to Etoposide WT IC50 = 56.33 ng/ml, *Bmal1-/-* IC50 = 110.9 ng/ml (C) and Daunorubicin WT IC50 = 45.35 nM, *Bmal1-/-*, IC50 = 95.5 nM) (D) compared to WT. *p< 0.01. Results are plotted as mean ± std. deviation (n= 5).

3. A.6 Increased sensitivity of *Bmal1*-deficient cells to oxidative stress is independent of p53.

Recently, the connection between circadian clock and p53 pathways was established. It was also reported that downregulation of BMAL1 results in impaired p53-dependent DNA damage response (144). Interestingly, as shown before our *Bmal1-/-* cells show enhanced resistance to DNA damaging agents such as Etoposide and Daunorubicin but more sensitivity to hydrogen peroxide. One of the possible mechanism for this difference in that the cell sensitivity could be effect of interference with p53 pathway. Therefore, we investigated the role of p53 in BMAL1-dependent response to oxidative stress. For this purpose, mouse embryonic fibroblasts overexpressing GSE56 (dominant negative fragment of p53 and can efficiently suppress p53 activity in different cell types) were used. WT GSE56 and *Bmal1-/-* GSE 56 were treated with hydrogen peroxide referring similar protocol similar to what was used before for lung fibroblasts.

Consistent with data from lung fibroblasts*, Bmal1-/-* GSE 56 cells were more sensitive to hydrogen peroxide–induced oxidative stress, compared with WT MEF56. This result indicates that suppression of p53 did not affect the difference in sensitivity to hydrogen peroxide between WT and *Bmal1-/-* cells. From this experiment, we concluded that increased sensitivity to oxidative stress in *Bma1-/-* cells is independent of p53 **(Figure16** *from Khapre R V et al. Cell cycle. 2011 Dec 1; 10 (23):4162-9).*

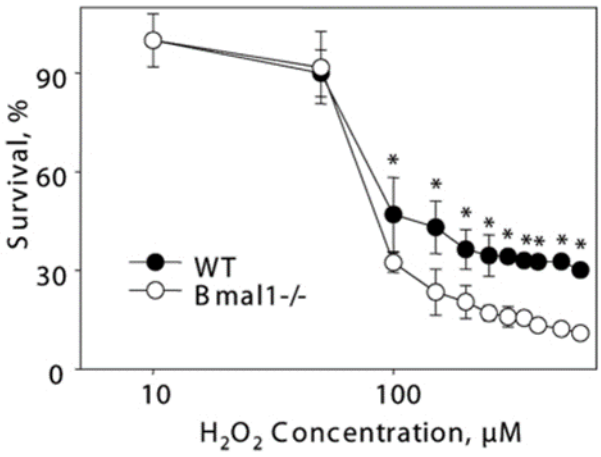


Figure 16 : Increased sensitivity of *Bmal1 deficient* cells to hydrogen peroxide is independent of p53.

WT GSE56 and *Bmal1-/-* GSE56 cells were plated at equal densities and treated with different concentrations of hydrogen peroxide for 72 hours. Cells were washed and fixed with 0.2% glutaraldehyde and stained with 0.05% crystal violet. The dye was extracted using 1% SDS and absorbance was measured at 570 nM. The absorbance value corresponds to the number of cells stained for each plate. The relative survival curve of GSE56 (dominant negative fragment of p53) WT (black circles) and *BMAL1-/-*(open circles) fibroblasts showed higher sensitivity of *Bmal1 -/-*cells to hydrogen peroxide compared with WT. Survival of untreated cells was set to 100%. (*) indicates $p<0.01$. Results are plotted as average of 3 independent experiments**.**

3. B. Circadian regulation of mTOR signaling pathway through BMAL1 dependent mechanism.

3. B.1 Cellular consequences of increased mTORC1 signaling due to *Bmal1* deficiency.

When we were passaging with WT and *Bmal1-/-* primary lung fibroblasts, we made unexpected results. Based on our senescence-associated β-Gal staining with tissues, we expected that *Bmal1-/-* primary fibroblasts would undergo senescence at earlier passages in comparison with WT. To our surprise, we did not observe any difference in the onset of replicative senescence between WT and Bmal1-/- cells. On the contrary, we observed that Bmal1-/- cells immortalized at earlier passages compare to WT. We also observed that the immortalized Bmal1-/- fibroblasts proliferated at a significantly higher rate (**Figure 17** from Khapre R V et al. Aging (Albany NY). 2014 Jan; 6(1):48-57).

We decided to investigate further on increased proliferation of Bmal1-/- immortalized cells. We also measured cell size and protein content in these Bmal1-/- cells. Cell size was measured by FACS wherein Bmal1-/- cells showed bigger cell size relative to WT (**Figure 18** from Khapre R V et al. Aging (Albany NY). 2014 Jan; 6(1):48-57). For measuring protein content, we used the Bradford assay. Immortalized lung fibroblasts were plated at an equal densities. After 6 hours of plating, cells were collected in PBS, counted and Bradford assay was performed using Coomasie blue (BIO-RAD) as per manufacturer's protocol. Bmal1-/- cells always showed higher protein concentration compared with an equal number of

WT cells (**Figure 19** from Khapre R V et al. Aging (Albany NY). 2014 Jan; 6(1):48-57).

The mTORC1 signaling pathway is an evolutionarily conserved pathway which acts as a sensor for nutrients, energy and growth factors for cells. Depending on the availability of these nutrients, it regulates various cellular processes such as cell growth, cell proliferation and metabolism. It has been shown that inhibition of mTORC1 pathway extends lifespan in many model organisms and confers protection against many age related pathologies. Recently, a role for the mTORC1 pathway in cellular senescence has been established. We found that *Bmal1-/-* cells demonstrate characteristics of increased activity of mTORC1 signaling such as increased cell proliferation, cell size and protein content**.**

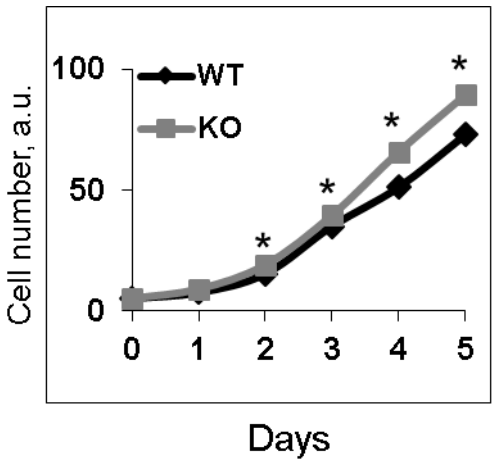


Figure 17 : *Bmal1* deficiency results in increased cell proliferation of lung fibroblasts.

Cell proliferation curves for WT and *Bmal1-/-* fibroblasts plated at equal density from 1 to 5 days in culture. Proliferation curve shows that after the second day of plating, *Bmal1-/-* cells start proliferating at significantly higher rate compared with WT, as indicated by the higher cell number. (*) $p < 0.05$. Results are plotted as mean $\pm$ std. deviation (n= 4 for each time point for both genotypes).

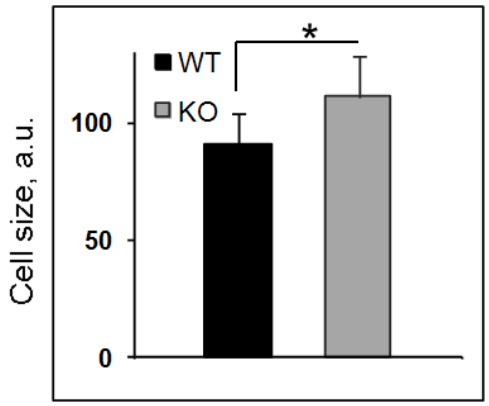


Figure 18 : *Bmal1* deficiency results in increase in cell size.

Cell size for immortalized WT and *Bmal1-/-* fibroblasts. As detected by FACS *Bmal1-/-* fibroblasts (grey Bar) shows significantly larger cell size compare with WT (Black Bar) (*) $p < 0.05$.

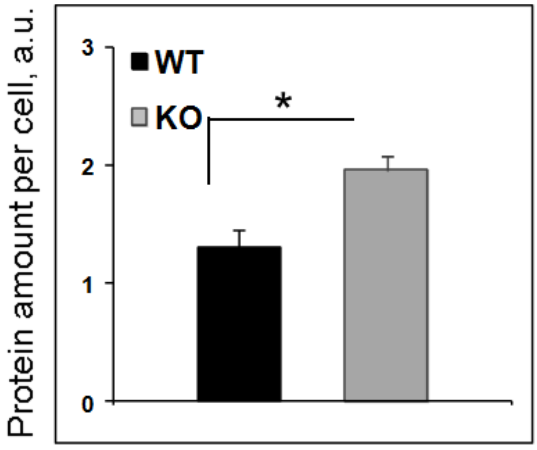


Figure 19 : *Bmal1* deficiency results in increased cellular protein content

Representative graph of protein content per WT (Black bar) and *Bmal1-/-* fibroblast (grey bar) measured by Bradford reagent. As shown *Bmal1-/-* fibroblast has significantly higher amount of protein in comparison with WT. (*) $p < 0.05$ Results are plotted as mean $\pm$ std. deviation from average of 3 independent experiments (n= 3 for both genotypes).

3. B.2 increased mTORC1 signaling results in increased cell proliferation, which is sensitive to mTORC1 inhibitor Rapamycin.

Since we know that *Bmal1-/-* cells show higher rate of proliferation, first we wanted to determine if this increased proliferation is a result of increased mTORC1 activity. If *Bmal1-/-* cells have increased mTORC1 activity, which leads to increased proliferation, then inhibition of mTORC1 activity should diminish the increased cell proliferation in *Bmal1-/-* cells. We used a well-known mTORC1 inhibitor, Rapamycin to inhibit its activity. Rapamycin is a bacterial antimetabolite and has antifungal activity. It binds to FKBP12 (FK506 binding protein) and this complex interacts physically with mTOR and inhibits mTORC1 activity. mTORC1 inhibition results in inhibition of cell proliferation.

For the cell proliferation experiment, cells were plated at an equal density in 96- well plates and treated with different concentrations of Rapamycin. Untreated *Bmal1-/-* cells proliferated at significantly higher rate compared with WT. Rapamycin treatment eliminated this difference (**Figure 20** from Khapre R V et al. Aging (Albany NY) 2014 Jan; 6(1):48-57). This indicates that increased proliferation of *Bmal1-/-*cells is a result of increased mTORC1 activity.

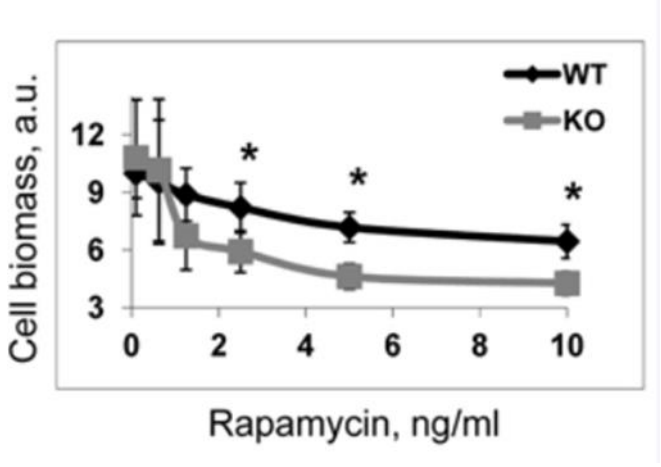


Figure 20: Increased proliferation of *Bmal1 deficient* cells is sensitive to Rapamycin.

Cell proliferation curves for WT(black line) and *Bmal1-/-* (grey line) fibroblasts plated at equal density and treated with media with indicated concentrations of Rapamycin for 72hr. Cell biomass was assayed by crystal violet incorporation. Proliferation curve indicates that Bmal1-/- fibroblasts are more sensitive to Rapamycin treatment. Data represent mean and standard deviation for 4 replicates. (*) indicates $p < 0.05$.

3. B.3 Increased mTORC1 signaling results in increased phosphorylation of mTOR downstream targets in *Bmal1* deficient immortalized lung fibroblasts and phosphorylation is sensitive to Rapamycin.

It is known that mTORC1 exerts its effects via activation of its downstream targets. mTOR is a serine/threonine protein kinase and it activates its downstream targets by phosphorylation. mTORC1 activity is measured by phosphorylation of its downstream targets. Though many mTORC1 targets are known, S6K1 (p70) is the best studied and commonly used target for mTORC1 activity. S6K1 is a direct target of and is phosphorylated by mTORC1 at a specific site T389. Ribosomal protein S6 is a direct target of S6K1 and is phosphorylated by S6K1 at four conserved residues (S235, S236, S240, and S244).Phosphorylation of ribosomal protein S6 is also a commonly used marker for mTORC1 activity. After observing the effect of Rapamycin on cell proliferation, we wanted to examine it at the molecular level. Therefore, we decided to determine phosphorylation of mTOR targets in WT and *Bmal1-/-* cells after Rapamycin treatment. We performed western blotting analysis to check expression of phosphorylated and total S6. Phosphorylation was detected using specific antibodies. As expected, we found that *Bmal1-/-* cells showed increased phosphorylation of S6 while total protein levels were similar in WT and *Bmal1-/-* cells. The increased phosphorylation of S6 in *Bmal1-/-* cells was sensitive to Rapamycin, indicating a higher mTOR activity in *Bmal1-/-* cells (**Figure 21** from Khapre R V et al. Aging (Albany NY). 2014 Jan; 6(1):48-57).

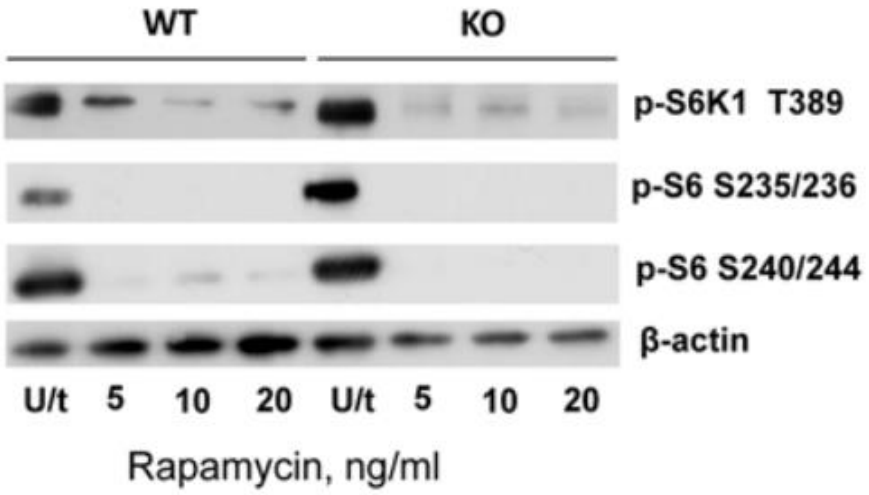


Figure 21 : Increased phosphorylation of mTORC1 targets in *Bmal1 deficient* cells is sensitive to Rapamycin.

Immortalized WT and *Bmal1-/-* cells were plated at equal density and either untreated (U/T) or treated with 5,10, and 20 nM Rapamycin for 24 hours and western blotting was performed with cell lysates using phosphorylated and protein specific antibodies. β-actin was used as a loading control to ensure equal loading. The above experiment is representative of two independent experiments.

3. B.4 Increased mTORC1 signaling results in increased phosphorylation of mTORC1 downstream targets in primary *Bmal1* deficient lung fibroblasts.

After confirming increased mTORC1 activity in immortalized *Bmal1-/-* fibroblasts, next we wanted to determine if we can detect increased mTORC1 activity in primary lung fibroblasts. We performed western blotting analysis on WT and *Bmal1-/-* lung fibroblasts for mTORC1 targets. We used 2 WT and 3 *Bmal1-/-* primary lung fibroblasts isolated from different mice. Each WT and *Bmal1-/- line* represents individual clone (**Figure 22** from Khapre R V et al. Aging (Albany NY). 2014 Jan; 6(1):48-57). Western blotting of different primary cells representing individual clone confirmed that mTOR activity is increased in all *Bmal1-/-* cells and is not limited to one specific clone. Twenty four hours before lysate collection, cells were incubated in either regular media or media with no serum. The no serum condition was used as a negative control because in the absence of nutrients or serum, the mTORC1 pathway is not activated, which can be observed by the low levels of phosphorylation of mTORC1 targets. We observed similar results in the western blot analysis.

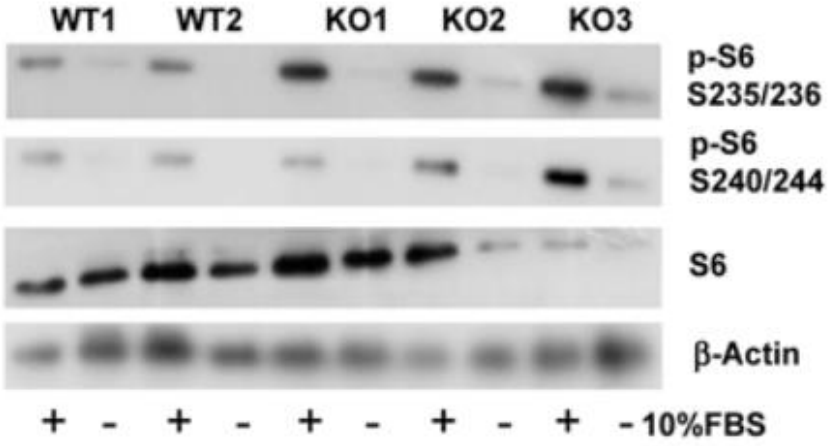


Figure 22 : *Bmal1-deficient* primary lung fibroblasts show high mTORC1 activity.

Western blotting of WT (WT-2T, WT-1F) and *Bmal1-/-* (KO-2T, KO-2L, KO-1F) primary lung fibroblasts incubated in media with (+) or without (-) 10% FBS using specific antibodies. *Bmal1-/-* cells show increased phosphorylation of S6K1 and S6 when compared with WT cells with no significant change in total protein levels. Treatment with 5, 10 and 20 nM Rapamycin for 24 hours resulted in decreased phosphorylation indicating high mTOR activity**.** B-actin was used as a loading control. The above result is representative of two independent experiments.

3. B.5 Liver tissues show oscillation in phosphorylation of mTORC1 targets over a period of 24 hours.

Having determined that *Bmal1* deficiency results in increased mTORC1 signaling in primary and immortalized lung fibroblasts supported our hypothesis that mTORC1 signaling is under circadian clock control. Next, we wanted to examine if we can observe similar relationship *in vivo*. If mTORC1 signaling is under circadian clock control, we expected that we should observe circadian rhythms in activation of mTORC1 signaling in tissues. To test this, we performed Western blotting analysis on different tissues collected from WT mice in period of 24 hr (Circadian profile).

For this purpose, we selected organs which are known to be under circadian clock control and are also reported to show robust mTORC1 activity. All tissues were collected over a 24 hr period starting at ZT2 (2 hours after lights were switched on) at 3 hours interval. All tissues were stored at -80˚C after collection. Lysates were prepared using sonication as described in material and methods and western blot was performed using phosphor-specific and protein- specific antibodies **(Figure 23).**As expected we observed that WT mice show oscillation in phosphorylation of mTORC1 targets namely S6K1 (T389), ribosomal protein S6 (S234, S235, S240, S244) and 4EBP1 (T37,T46) with no rhythms in total protein expression levels.

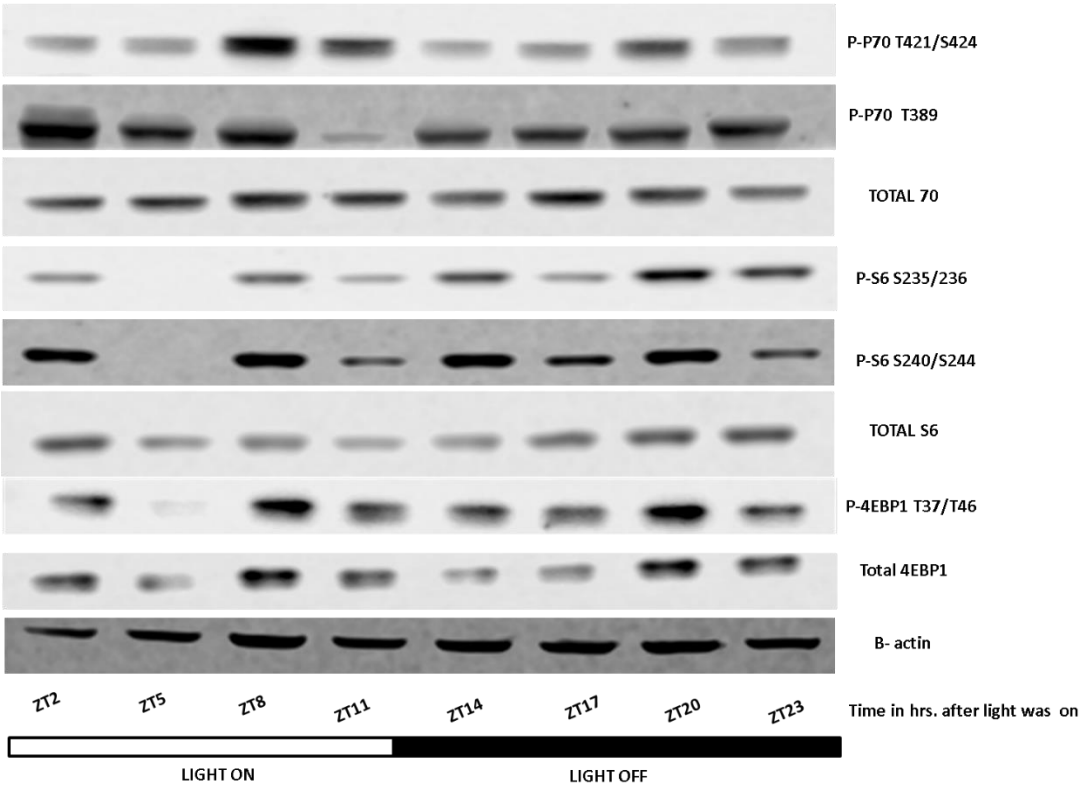


Figure 23 : mTORC1 signaling shows oscillations in activation over a period of 24 hour in liver.

Western blotting of liver of WT mice collected over 24 hours (ZT2 to ZT23) at 3 hours interval. Lysates were prepared by sonicating tissues in Cell Signaling Buffer. Blots were incubated with specific primary antibodies as shown in figure. β-actin was used as a loading control. Liver tissue shows oscillation in the phosphorylation of S6K1, S6, and 4EBP1 without significant changes in total protein levels.

3. B.6 Heart tissues show oscillation in phosphorylation of mTORC1 targets over a period of 24 hours.

After having determined that liver shows oscillation in mTOR activity, next we wanted to determine if this effect is specific to liver or can be detected in other tissues. Next we decided to mTOR activity in the heart, another organ which is very well known to be under circadian control. For this purpose, we collected tissues in similar way as described for liver. Western blot analysis was performed using specific antibodies for the mTORC1 signaling pathway As indicated in figure, heart did show rhythmic activation of mTORC1 signaling but it is not as robust as liver, indicating tissue variability (**Figure 24).**

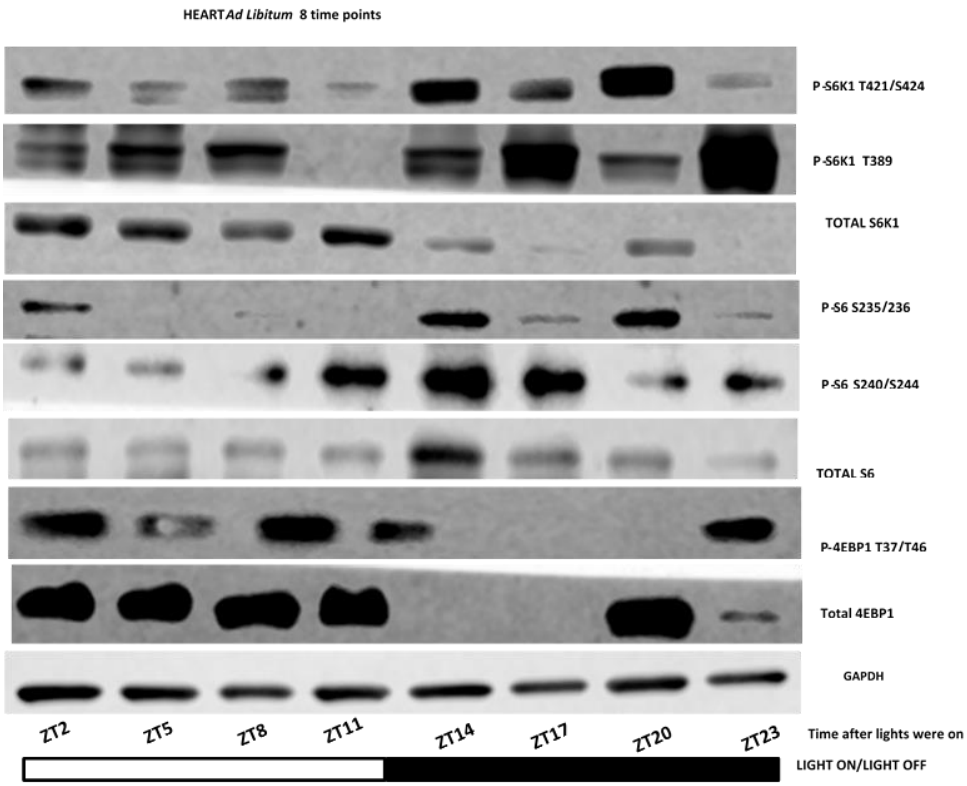


Figure 24 : mTORC1 signaling shows oscillations in activation over a period of 24 hours in the heart.

Western blotting of heart tissues of WT mice collected over 24 hours (ZT2 to ZT23) at a 3 hours interval. mTORC1 downstream targets were detected using antibodies recognizing the indicated proteins (total or phosphorylated). Heart shows oscillation in the phosphorylation of S6K1, S6, 4EBP1 without significant changes in total protein levels. GAPDH was used as a loading control.

3.B.7 Spleen show oscillation in phoshorylation of mTORC1 targets over period of 24 hours.

We also determined rhythms in activation of mTORC1 signaling in the spleen. Tissues were collected as described for liver and heart. We observed a similar pattern of rhythms in spleen **(Figure 25).**

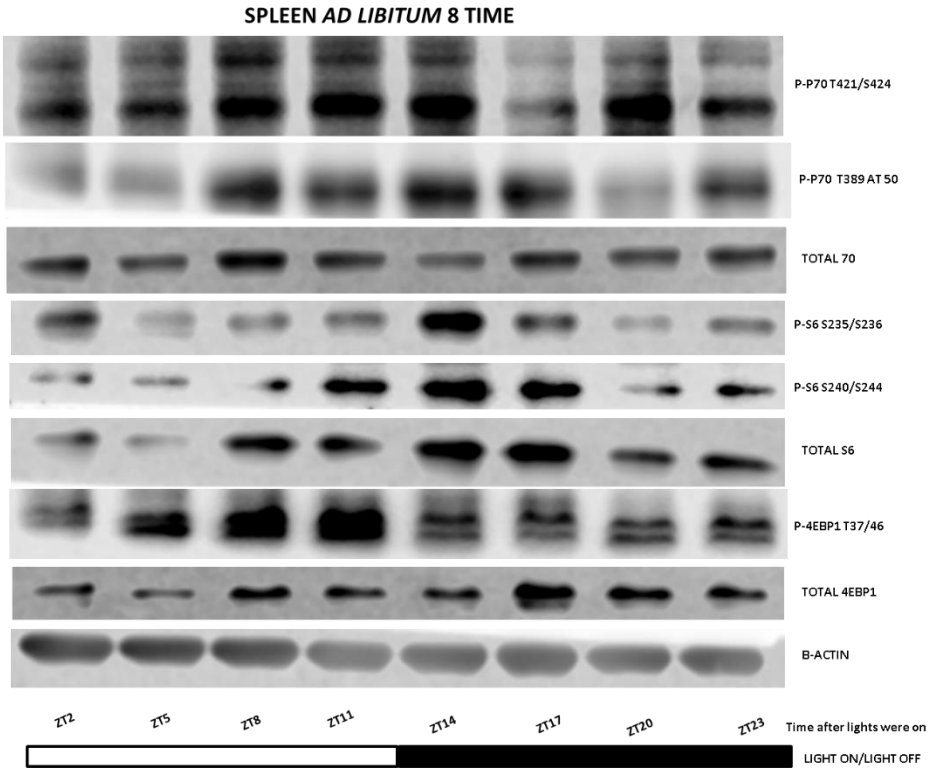


Figure 25 : mTORC1 signaling shows oscillations in activation over a period of 24 hours in spleen tissue.

Western blotting of spleen tissues of WT mice collected over 24 hours (ZT2 to ZT23) with 3 hours interval. β-actin was used as loading control. As shown in figure, spleen shows oscillation in the phosphorylation of p70, S6, 4EBP1 without significant changes in total protein levels.

3. B.8 Daily rhythms in activation of mTORC1 signaling in liver under time restricted feeding regimen.

mTORC1 signaling pathway is a nutrient sensing pathway and food intake results in its activation. Since liver is the central organ for metabolism, mTORC1 activity in liver can be significantly affected by food. For all our above experiments food was available all the time *(Ad libitum)* to animals and it is possible that the oscillations in activation of mTOR were result of different eating habits of individual animals.

To confirm our hypothesis that mTORC1 signaling is under control of circadian clock, we changed the feeding regimen for mice from *Ad Libitum* to time restricted feeding (tRF) regimen. In time restricted feeding, 95-100% of their daily intake consumption was provided at ZT14 (approximate time when mice wake up) and no food was provided for next 24 hours. We observed that the total amount of food provided was consumed within the first 3-4 hours. All mice were kept with tRF for a 2 weeks regimen before collection of tissues. We also analyzed daily in cage activity of these mice to check effect of changed food regimen on their behavior. We found no difference in the behavior for these mice when kept on *Ad libitum* or time-restricted feeding regimen. Tissues were collected as described before and stored at -80˚C. Lysates were prepared as per previous protocol and western blot analysis was performed. As expected, after food was provided at ZT14 we observed a gradual increase in phosphorylation of S6K1 at T389, which reached maximum at ZT22 and then decreased gradually. We also observed a similar pattern in phosphorylation of 4EBP1 with delayed onset indicating different

regulation of S6K1 and 4EBP1 by mTORC1. Rhythms in phosphorylation were consistent even for S6. Interestingly at ZT10 and ZT14 when no food was available, we observed an increase in phosphorylation of S6K1 at T389 indicating anticipation of food **(Figure 26).**

We observed that liver tissues from animals with a time-restricted feeding regimen showed oscillations in mTORC1 activation, as observed with *Ad Libitum* mice. These oscillations were even more robust and clear compared with *Ad Libitum* indicating a masking effect of food in *Ad Libitum* and that effect is eliminated in time-restricted feeding. Increased phosphorylation of mTORC1 targets at the time of food availability indicates activation of mTORC1 signaling.

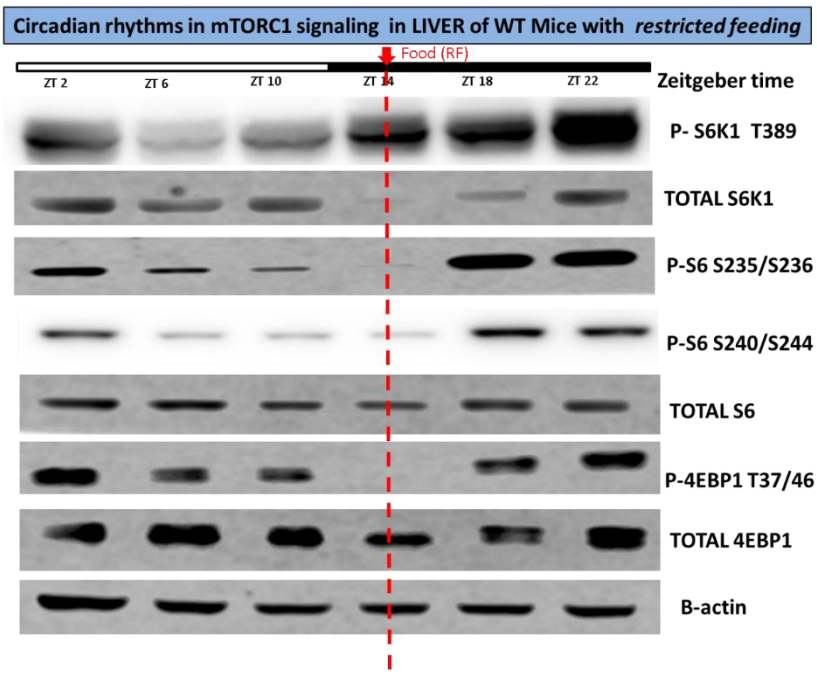


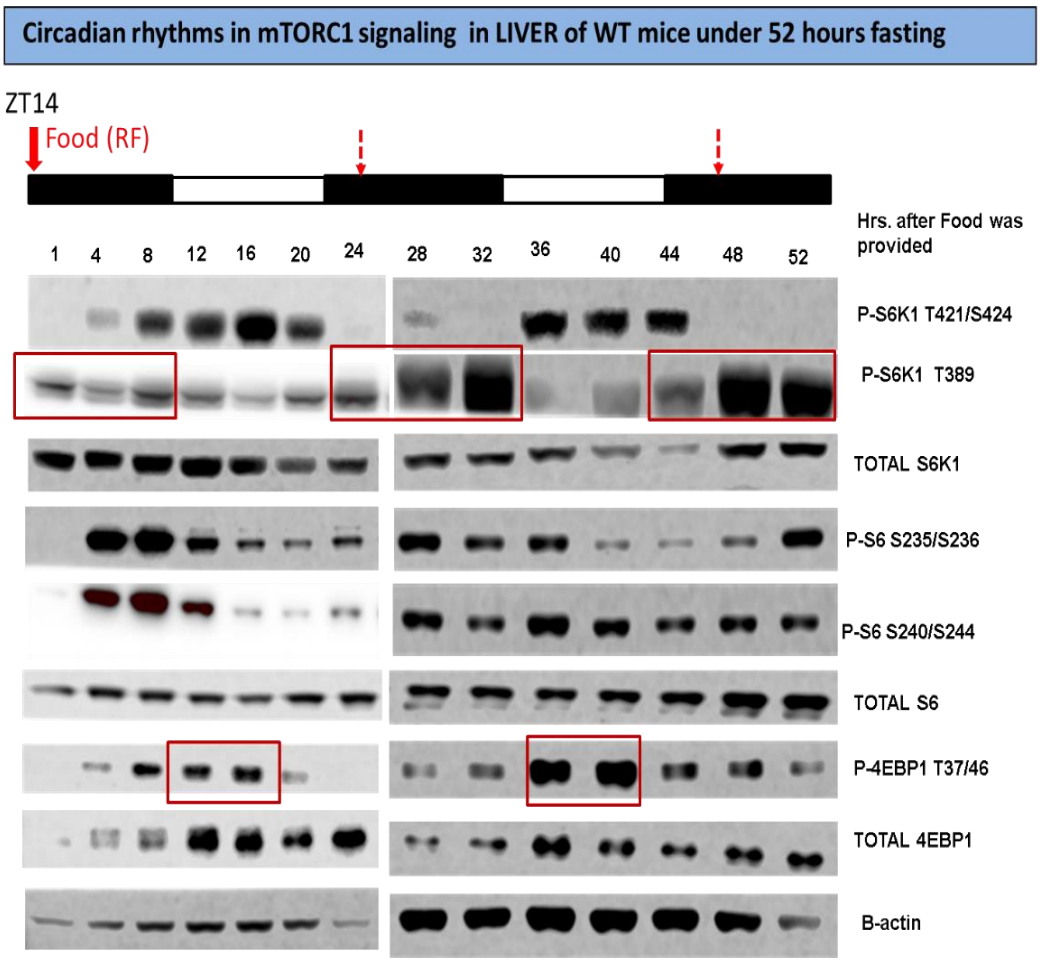
Figure 26 : time restricted feeding demonstrates clear rhythms in activation of mTORC1 signaling in the liver tissue.

Western blot of liver tissues collected from WT mice fed at ZT 14 with 100% of daily food intake. Tissues were collected over 24 hours starting at ZT14 with 4 hours interval. At ZT 14 phosphorylation of S6K1 at T389 started to increase until ZT 22 and then decreased as indicated by low levels at ZT 2 and ZT6. At ZT14 even though food was not provided, phosphorylation of S6K1 at T389 showed an increase, indicating anticipation of food. β-actin was used as loading control.

3. B.9 Daily rhythms in activation of mTORC1 signaling in liver are endogenous and independent of food.

Once we confirmed that under tRF regimen mTORC1 displays rhythmic activation, next we wanted to check if this rhythmicity is endogenous and can be sustained independent of food. For this purpose, we used 52 hours fasting conditions for animals. For fasting experiments, the same amount of food as tRF was provided at ZT14 and then no food was provided for the next 2 feeding cycles (48 hours). Tissues were collected and stored as described before. Western blot analysis was performed as described earlier. We observed that both S6K1phosphorylation sites, T421/S424 and T389 show rhythms, but they were in different phases.T421/S424 is not mTORC1 specific phosphorylation site and is known to be activated by MAPK. But T389, mTORC1 specific phosphorylation site showed activation at ZT14-ZT22 periodically starting from 1hr, 24hrs, and 48hrs after the food was provided. 4EBP1 phosphorylation also showed similar pattern of rhythms but with delayed onset occurring at ZT18 (4 hr after food was provided) and repeated every 24 hr **(Figure 27).** Even when no food was provided at time points when it was expected such as at time points 24 hr and 48 hr, we observed increased phosphorylation of S6K1 at T389, indicating anticipation of food. It shows that rhythmic activation of mTORC1 signaling is endogenous and independent of food. This result supported our hypothesis that mTOR pathway shows endogenous oscillations in activation. These oscillations in mTOR activation are independent of food and nutrients and are probably driven by circadian clock.

A.


Circadian rhythms in mTORC1 signaling in LIVER of WT mice under 52 hours fasting
ZT14
Food (RF)
Hrs. after Food was provided
1 4 8 12 16 20 24 28 32 36 40 44 48 52
P-S6K1 T421/S424
P-S6K1 T389
TOTAL S6K1
P-S6 S235/S236
P-S6 S240/S244
TOTAL S6
P-4EBP1 T37/46
TOTAL 4EBP1
B-actin

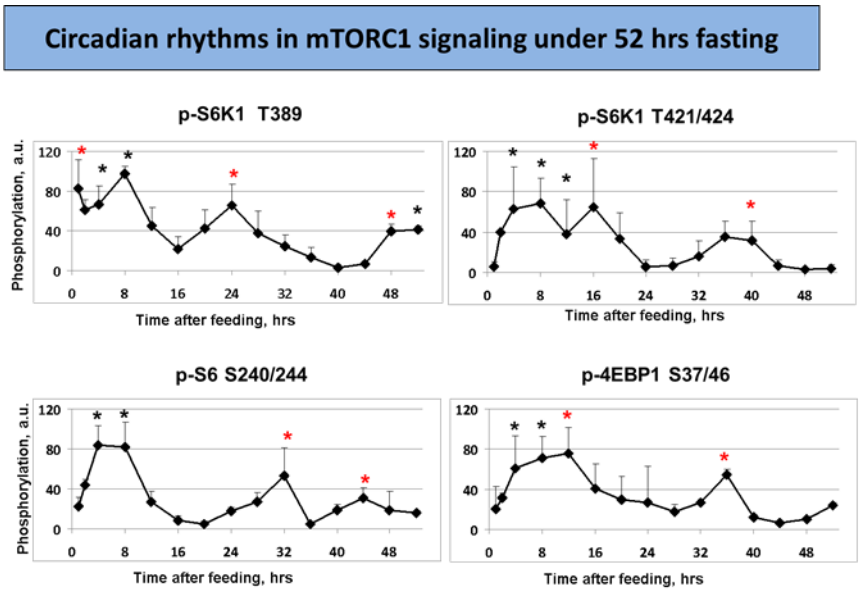


Figure 27 : Oscillations in activation of mTORC1 signaling in liver tissues are endogenous and independent of food.

A. Western blotting for mTORC1 downstream targets in WT liver. At 0 hr, 24 hr, 48 hr after food was provided At 24 and 48 hr, phosphorylation at T389 was increased even when no food was provided. 4EBP1 also shows similar pattern of phosphorylation but with delayed onset. No significant changes in total protein levels were observed. GAPDH was used as loading control. **B.** Quantification shows circadian oscillations in phosphorylation of S6K1, S6 and 4EBP1. Peaks marked with red asterisks indicate maximum phosphorylation. For S6K1, phosphorylation at T389 (mTORC1specific site) shows 3 asterisks at 0hr,24 hr and 48 hr indicating oscillations in phosphorylation with periodicity of 24 hrs. Phosphorylation at T421/S424 does show a 24 hr periodicity but is in anti-phase with T389. 4EBP1 also shows 2 peaks in phosphorylation that are delayed

compared to S6K1 and occurring with a periodicity of 24 hr. Data represent mean and standard deviation for 3 replicates (*) $p < 0.05$.

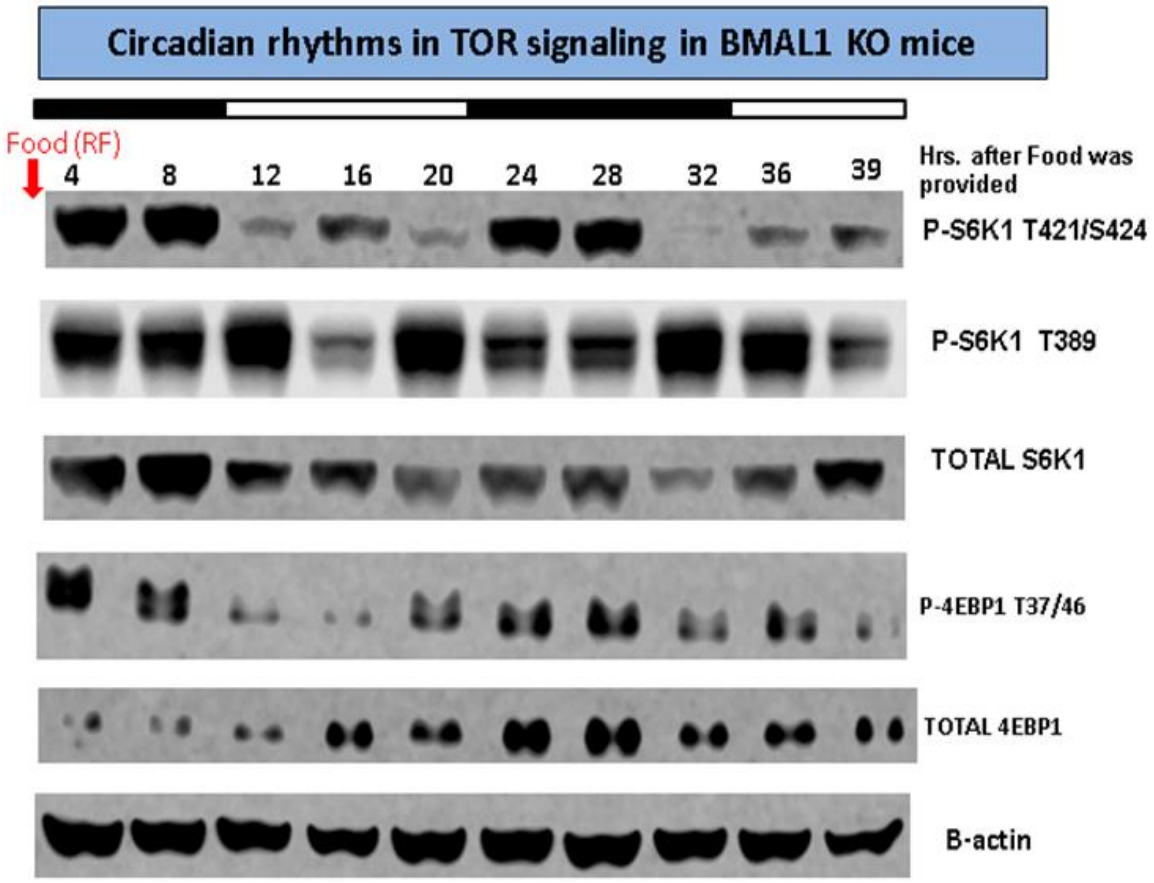
3. B.10 *Bmal1* deficiency (disruption of circadian clock) results in increased mTORC1 signaling and change in phase of activation of mTORC1 *in vivo*.

Having determined that *Bmal1* deficiency results in increased mTORC1 signaling in primary and immortalized lung fibroblasts and different tissues showed oscillations in activation of mTORC1 signaling, we next hypothesized that *Bmal1* deficiency would result in alteration in mTORC1 activity in different tissues. The alternation could be a change in level of phosphorylation, change in phase of activation of downstream targets or change in amplitude of oscillation. We also hypothesized that tissues which are known to be under circadian clock control will be affected most by *Bmal1* deficiency. To test this hypothesis, we decided to perform western blotting analysis on liver tissue from BMAL1 KO mice. We checked mTORC1 signaling in BMAL1 deficient mice kept on time restricted feeding regimen. We followed a similar food regimen as in the WT mice used before.

As expected, we observed non-rhythmic phosphorylation of mTORC1 targets S6K1 (T389) and 4EBP1 (T37/46) indicating an effect of *Bmal1* deficiency on regulation of mTORC1 signaling. In addition we also observed significantly increased phosphorylation of S6K1 at T389 for many time points, indicating a continuous activation of mTOCR1 signaling. Phosphorylation at T421/S424 (non mTOR specific site) on S6K1 did not show significant change in oscillations or in level of phosphorylation, indicating *Bmal1* deficiency results in deregulation of mTORC1 signaling specifically **(Figure 28)**. It results in constitutive activation of mTORC1 signaling in BMAL1-deficient mice, supporting our hypothesis that the

circadian clock negatively regulate mTORC1 signaling through a BMAL1-dependent mechanism.

A.


Circadian rhythms in TOR signaling in BMAL1 KO mice
Food (RF)
4
8
12
16
20
24
28
32
36
39
Hrs. after Food was provided
P-S6K1 T421/S424
P-S6K1 T389
TOTAL S6K1
P-4EBP1 T37/46
TOTAL 4EBP1
B-actin

B.

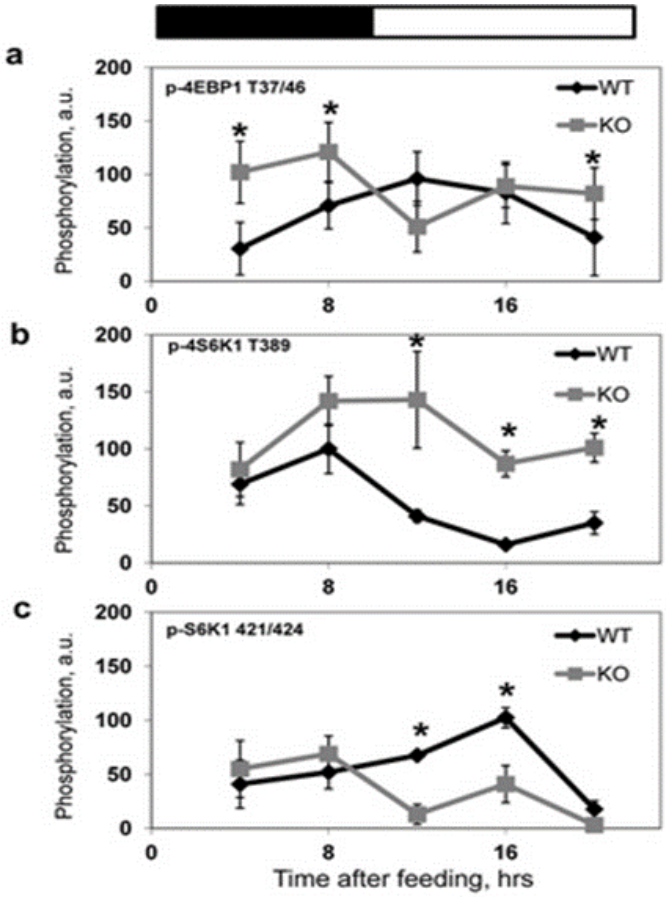


Figure 28 : *Bmal1* deficiency results in non-rhythmic activation of mTORC1 signaling in liver.

A. Western blot of liver tissues collected from BMAL1 mice over 40 hours starting at ZT14 with 4 hours interval. Blots were incubated with specific primary antibodies as shown. B-actin was used as loading control. Bmal1-/- mice show nonrhythmic oscillations in the phosphorylation of S6K1 at T389 (mTOR site) which is constitutively active at almost all time points. In contrast, phosphorylation at T421/S424 (non mTOR site) does not show significant difference in oscillations

and level of phosphorylation. 4EBP1 also shows disrupted pattern of phosphorylation in BMAL1 deficient mice.

Quantification of phosphorylation of S6K1 (T389), 4EBP1 (T37/46) and S6K1 (T421/S424) in WT (Black line) and BMAL1 KO (Grey line) liver. Data represent mean and standard deviation for 4 replicates. (*) $p < 0.05$ Figure *from Khapre R V et al. Aging (Albany NY). 2014 Jan; 6(1):48-57*

3. B.11 Regulation of mTORC1 signaling at mRNA level by BMAL1 as transcription factor.

Finally, in an effort to determine, how the circadian clock, especially BMAL1 regulates mTORC1 signaling, we used quantitative PCR approach. Considering that BMAL1 is a transcription factor, we expected to observe regulation at mRNA level. mTORC1 signaling is regulated either at upstream (activators and inhibitors) level or at mTORC1 complex level (different components of the complex). Therefore, we decided to check mRNA expression of mTOR complex and mTOR pathways genes. We found that expression of *tor* and *deptor* mRNA was highly affected by *Bmal1* deficiency. In support of our hypothesis that circadian clock negatively regulates mTORC1 signaling, we observed that expression of *tor* was significantly upregulated at several time points **(Figure 29a)**, while expression of *deptor* (negative regulator of mTOR) was significantly downregulated at several time points **(Figure 29b)** in the liver of *Bmal1-/-* mice.

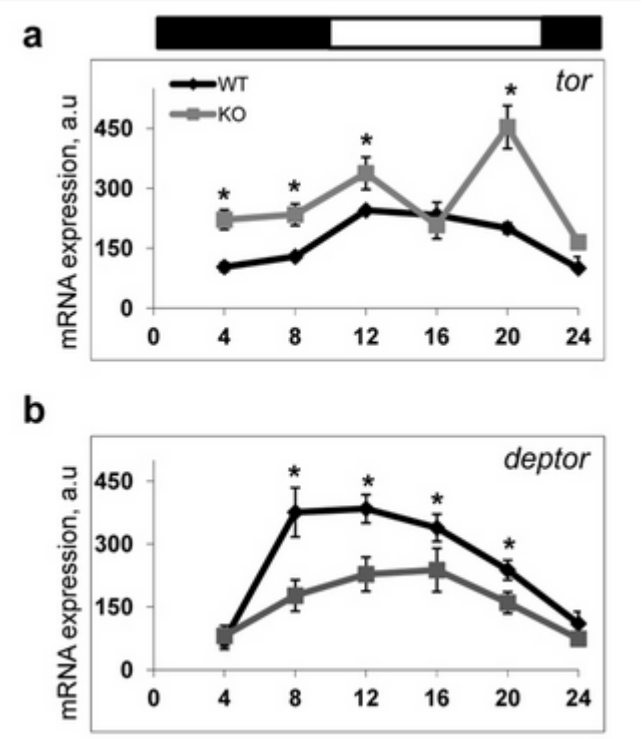


Figure 29 : mRNA expression of *mtor* and *deptor* in liver from WT and BMAL1 KO mice.

The mRNA expression of *tor* (a) and *deptor* (b), in liver of tRF WT ((black diamonds) and BMAL1 KO (grey squares) mice were analyzed over a daily cycle (24 hours) by real-time RT PCR. All data was normalized to 18s ribosomal RNA expression. Bars on top of the figure represent the light (open bars) and dark (black bars) part of the day. Data represent mean and standard deviation for 3 replicates. $P < 0.05$. (*) Statistically significant difference.

CHAPTER IV

DISCUSSION

4.1 Overview

Over the last century, through progression in drug development, improvement in living conditions and increased health awareness, human life expectancy has increased dramatically. As a result, a substantial percentage of population lives significantly longer than in the past. With increasing human lifespan, we have to cope with growing problems and difficulties due to age related pathologies. These pathological conditions not only affect the quality of life but also affects economic status. Therefore, elucidation of mechanisms involved in age related pathologies are becoming extremely important with eventual goal of developing effective and preventive therapies.

For centuries, humans have been looking for ways to avoid aging, which gave rise to a foundational need for understanding the process of aging. Albeit several proposed theories, the mechanisms of aging are not well understood. As inevitable

as it is, aging is a complex process and can be defined in many ways. In simple terms, it involves deterioration of biological systems with reduced fitness and over all well-being of living organisms. It can also be seen as a risk factor for causing new diseases or consequence of many diseases.

Development of various aging models has made remarkable progress in the field. Our lab is also working with one of the models of premature aging in mice. This model represents disrupted circadian clock due to deficiency of core circadian clock protein BMAL1. The circadian clock is an endogenous time keeping system in organisms which helps them synchronize their physiology and behavior with environmental changes surrounding them such as day-night cycle. Circadian clock creates rhythms in various physiological processes. In mammals, it is represented by an autoregulatory negative feedback loop of core clock proteins. BMAL1 and CLOCK are transcription factors which heterodimerize and bind to E- box elements in promoter region of many genes to drive their expression. Circadian clock also drives expression of genes which are not part of the loop but their rhythmic expressions are important for physiological outputs (1, 2, 3)

The area of circadian clock has been explored for over 45 years. It was discovered that administration of mutagenic substance in fruit flies resulted in 3 mutants with different circadian cycles (142). After 25 years of the first discovery, the first mammalian circadian clock gene CLOCK was discovered in mice leading to discovery of other core clock genes required for circadian rhythms. In recent years, extensive research in circadian clock field has linked it to many sleep, neurological, cardiovascular, and metabolic disorders (17,35). Even certain types

of cancer have been linked to disruption of circadian clock. It was demonstrated in various studies that shift workers (clock is disrupted) have higher risk of developing cardiovascular, metabolic, neurological disorders and cancer. Considering noteworthy percentage of world's population works in shifts, study of disrupted circadian clock is getting even more emphasis especially to identify role of clock proteins in aging and age related pathologies. Various animal models of disrupted circadian clock are available which exhibit conditions that are related to different aging pathologies.

Our lab focus has been deeply invested to determine molecular mechanisms involved in premature aging in BMAL1 (core Clock component) deficient mice. BMAL1 is the only known circadian clock component, which in deficiency, causes complete arrhythmicity in constant darkness in mice. Our lab has previously demonstrated that *Bmal1-/-* mice display reduced lifespan. They also develop many age related pathologies such as cataract, memory loss, sarcopenia and infertility prematurely. Our lab has previously shown that BMAL1 plays a significant role in Reactive Oxygen Species (ROS) homeostasis. *Bmal1* deficient *mice* displayed increased accumulation of ROS in different organs such as heart, kidney and spleen. Interestingly, similar tissues also displayed age dependent reduction in size. In addition to that, *Bmal1-/-* mice treated with antioxidant N-acetyl-L-cysteine through their lifespan showed increased average and maximum life span. Treatment with antioxidant also reduced certain age related pathologies such as cataract and weight loss but surprisingly had no effect

on many other pathologies observed in *Bmal1-/-* mice. This indicated that ROS can affect aging but ROS independent mechanisms are involved.

It has been documented that aging is associated with increased accumulation of senescent cells in tissues. Senescence is a specific state of cells characterized by irreversible growth arrest, flat and enlarged morphology, changed gene expression, resistance to apoptosis and secretion of degradative enzymes which are believed to disrupt surrounding tissue environment and might contribute to aging. Senescence is categorized into 3 different types; 1) Replicative,2) Stress induced,3) Oncogene induced. Though senescence was first reported as replicative senescence in cell culture, its significance in age associated senescence is not clear. Conversely, other research shows that stress induced senescence plays a role as anticancer mechanism and can be related to mechanisms of aging. We further investigated role of different types of senescence in *Bmal1* deficient mice. Interestingly tissues from *Bmal1-/-* mice showed significantly higher number of senescent cells when compared to WT mice of same age. These observations drove us to further investigate molecular mechanisms involved in role of BMAL1 in cellular senescence which contribute to aging observed in *Bmal1-/-* mice. Results presented in this dissertation present different mechanisms with role of BMAL1 in premature aging in mice. In this proposal, we identified that BMAL1 regulates oxidative stress induced senescence which is in correlation with our previously published data displaying disrupted ROS regulation in *Bmal1* deficient mice. In this thesis, we demonstrated that there is no significant difference in replicative senescence between WT and *Bmal1-/-* primary fibroblasts

in vitro. This also indicates BMAL1 does not contribute to replicative senescence and also replicative senescence might not contribute to premature aging in *Bmal1-/-* mice.

Oxidative stress is a potent stress inducer and probably has role in stress induced senescence in vivo. We demonstrate here that BMAL1 plays a role in oxidative stress sensitivity and oxidative stress induced senescence. BMAL1 deficiency results in increased oxidative stress in mice. Recent studies with shift workers, demonstrated increased oxidative stress measured by oxidative stress markers. As shift work is known to disrupt circadian clock, these results very well support our observations of increased oxidative stress in BMAL1 KO mice (142).

Our results pointed to a new question if the sensitivity of *Bmal1-/-* cells is specific to oxidative stress or they are also sensitive to genotoxic stress. Role of BMAL1 in cell sensitivity to genotoxic stress and cell cycle is well documented (our review). We checked effect of BMAL1 deficiency in genotoxic stress using different DNA damaging agents each with different mode of action. Previous studies suggest, tumor cells with downregulation of Bmal1 expression with shRNA were more resistant to Etoposide induced apoptosis (143). In agreement with this data, we observed that *Bmal1-/-* cells show more resistance to some DNA damaging agents such as Etoposide or Daunorubicin. But no difference was observed with another DNA damaging agent named 5-flurouracil. Other research suggests that BMAL1 interferes with p53 dependent DNA damage response (144). However, we further observed that role of BMAL1 in oxidative stress response was independent of p53. After collective analysis of this data, we revealed that BMAL1 does not play any

role in replicative senescence but it regulates oxidative stress induced senescence *in vivo*. *Bmal1* deficiency results in accumulation of senescent cells *in vivo* which can contribute to premature aging. Our work is supported by a recently published work on role of *Bmal1* in neuronal oxidative damage and neurodegeneration suggesting its role brain aging (145).

It is well known that, senescent cells display over activation of growth promoting pathways such as mTORC1. mTORC1 is a nutrient sensing pathway involved in various cellular processes such as cell growth and proliferation. Many growth related processes such as nutrient uptake, protein synthesis, ribosome biogenesis are regulated by mTORC1. In senescent cells, though cell cycle progression is arrested, cell growth is not. Therefore, increased mTORC1 activity results in senescent phenotype such as enlarged morphology. In immortalized cells, where cell cycle is not arrested, increase in mTORC1 activity can result in increased proliferation rate. Consistent with previous data, *Bmal1*-/- deficient cells have significantly increased proliferation rate, protein content, cell size and cell biomass compared to WT. This hinted us for overactivation of mTORC1 in Bmal1 deficient mice.

mTOR acts as a sensor of nutrients and regulator of cellular processes, its role underlines its importance in metabolism, body homeostasis and overall well-being of organisms. Deregulation of mTORC1 signaling is linked to various metabolic disorders, cancer and aging. Genetic mutations and pharmacological inhibition of mTORC1 signaling in various models have been shown to extend lifespan which suggests mTORC1 is a key regulator of aging. Though mTORC1

regulation under nutrients or stress conditions have been studied, its regulation under physiological conditions is unknown.

In our present work, we demonstrate for the first time that mTORC1 is regulated by circadian clock under physiological conditions. Considering mTORC1 is a nutrient sensor, and nutrients are available only at feeding (wake cycle) dictated by circadian clock, we hypothesized that mTORC1 might be controlled by circadian clock. This regulation can help organisms to synchronize food availability with anabolic processes such as protein synthesis, lipid biosynthesis which are under mTORC1 control. The connection between circadian clock and mTORC1 has never been explored before. Results presented here illustrate circadian regulation of mTORC1 and effect of disrupted circadian clock on mTORC1 signaling. We demonstrated that mTORC1 is rhythmically activated as confirmed by phosphorylation of its downstream targets. This rhythmic activation is independent of food suggesting presence of endogenous regulation supporting our hypothesis. The next question was if mTORC1 is under circadian control, then how does disrupted circadian clock affects mTORC1 signaling? In *Bmal1* deficient mice, we observed that mTORC1 activation is non rhythmic and even elevated at several different time points. This indicates that circadian clock negatively regulates mTORC1 and disruption of circadian clock results in overactivation of mTORC1 signaling. Thus our current data suggests that constitutively elevated mTORC1 signaling may result in premature aging in Bmal1 deficient mice. To test this hypothesis, our collaborators checked whether pharmacological suppression of mTORC1 by Rapamycin (potent and specific mTORC1 inhibitor) can extend

lifespan in *Bmal1* deficient mice. Previous studies with Rapamycin to inhibit mTORC1 demonstrated increased life span and delayed onset of age related pathologies in old mice. In support with this, our collaborators demonstrated that lifelong treatment with Rapamycin extended lifespan of *Bmal1* deficient mice. This work is still in progress.

During our experiments with time restricted feeding and fasting regimen, we made interesting observations of differential regulation of S6K1 and 4EBP1 phosphorylation by mTORC1. S6K1 is phosphorylated at earlier time point (ZT14) when compared with 4EBP1 (ZT18). Both S6K1 and 4EBP1 function as translation regulators.

Eukaryotic initiation factor 4E (eIF4E) is a mRNA 5' cap binding protein and part of eukaryotic initiation factor 4F (eIF4F) complex involved in a cap dependent translation (146). In the absence of growth factors or nutrient signals, hypophoshorylated 4EBPs associate with eIF4E tightly, inhibiting assembly of eIF4F complex. In the presence of nutrients and growth factors, 4EBPs are phosphorylated by mTORC1 at multiple sites resulting in its dissociation from the eIF4E (110). Many targets of S6K1 like ribosomal protein S6, SKAR, mRNA splicing factor (147), eIF4B, which is activator of eIF4A helicase (part of eIF4F complex) (148), PDCD4, an inhibitor of eIF4A (149) are also part of translation machinery. If both S6K1 and 4EBP proteins are involved in translation, then why are they phosphorylated at different time points? What is the significance of this differential phosphorylation? Recent studies demonstrated that, eIF3-preinitiation complex (eIF3-PIC) acts as dynamic scaffold for S6K1 and mTOR/Raptor

(150,151). This binding is sensitive to growth factors and nutrient signals and Rapamycin. Under unstimulated conditions, inactive S6K1 is associated with eIF3-PIC complex, whereas mTOR/Raptor binding to the complex is weak. When stimulated by nutrients and mitogens, mTORC1 associates with eIF3-PIC complex and phosphorylates S6K1 at T389. This phosphorylation at T389 results in dissociation of S6K1 from eIF3-PIC complex and S6K1 can phosphorylate its targets involved in translation present in close proximity. This is also observed in our experiments where phosphorylation of ribosomal protein S6 occurs later after phosphorylation of S6K1. The study also demonstrated the constitutive presence of eIF3 complex and eIF4E at the cap structure whereas, after insulin stimulation recruitment of eIF4G and dissociation of 4EBP1 was time dependent. This indicates that eIF3 might interact with eIF4E. The signal stimulated mTOR/Raptor association with eIF3 helps phosphorylation of 4EBP1 resulting in its dissociation from eIF4E. The suggested model indicates that association of S6K1 with eIF3 might help for its early phosphorylation by mTORC1 (150,151).

Finally, we illustrated the mechanism of mTORC1 regulation by circadian clock. During our experiments, we observed that mTOR itself and DEPTOR (negative regulator of mTOR) show oscillation in their mRNA expressions, indicating transcriptional control of circadian clock which is known to be common mechanism for regulation of many clock controlled genes. We also propose a model underlying physiological significance of this regulation. During wake cycle, organisms are active and they engage in feeding making nutrients available. This activates mTORC1 inducing many anabolic processes such as protein synthesis, lipid biosynthesis and cellular growth. But during sleep phase of cycle, when organisms don not eat, it is essential to inhibit anabolic processes and encourage catabolic processes to break down macromolecules to make nutrients available. mTORC1 activates anabolism and inhibits catabolism, so by switching mTORC1 activity to on or off we can regulate anabolic and catabolic processes. This manuscript presents critical role of circadian clock in switching off mTORC1 activity during sleep phase of daily cycle.

Although mTORC1 is critical during development, it's over activation could detrimental in adult organisms. Aging is associated with decline in Circadian rhythms and metabolic homeostasis. We present here negative regulation of mTORC1 signaling by circadian clock. Over activation of mTORC1 signaling in disrupted circadian clock model (BMAL1 deficient mice) can result in constitutive activation of anabolic processes leading to nutrient depletion, imbalance in homeostasis which can contribute to accelerated aging observed in Bmal1-/- mice.

4.2 Future directions

Another interesting observation that we made was the increased rate of cell proliferation of immortalized *Bmal1-/-* fibroblasts. Cell proliferation is mainly regulated by cell cycle, a process that occurs in series of stages namely G1, S, G2, and M leading to cell division. Cell cycle is tightly regulated at all these stages by many cell cycle regulators and surveillance mechanisms known as cell cycle checkpoints. Cell cycle checkpoints monitor progression of cell cycle and can sense DNA damage through interacting pathways. There are 3 main checkpoints in cell cycle: G1/S transition, S stage progression and G2/M transition. Upon DNA damage or failure of previous activity in cell cycle, these check points interrupt cell cycle to provide time for repair DNA damage. This leads to delayed cell cycle progression or cell cycle arrest (152,153).

In recent years, many studies with the circadian clock genes mutations have demonstrated the connection between the circadian clock and the cell cycle. In unicellular organisms, restriction of DNA synthesis to nighttime for protection from UV damage suggested a possible control of cell cycle by the circadian clock (154). Studies with microarray analysis and measurement of cell cycle genes expression have reported potential targets for this regulation at various stages of cell cycle. Some of them are discussed below.

The transcription factor *c-myc* is important regulator of G1 stage. Reporter assay studies demonstrated that *c-myc* is a clock controlled -gene. The bmal1/NPAS2 (clock homolog) complex transcriptionally repress c-myc promoter (154). One of the important target gene of c-myc is *Gadd45α* which is involved in cell growth

arrest. *Gadd45α* is negatively regulated by *c-myc*. Therefore, disruption of the circadian clock can result in upregulation of *c-myc* and thereby downregulation of *Gadd45α* resulting in continuous cell cycle progression (155,156). The BMAL1/CLOCK complex also directly regulates expression of *Wee 1* kinase that regulates G2-M transition. Wee1 kinase inactivates the cdc2/cyclin B1 complex by phosphorylation. It results in cell cycle arrest at G2-M interface. Wee1 Kinase is also activated in response to DNA damage (157). Additional many cell cycle genes involved in G1-S and G2-M regulation such as *Cyclin B1*, *Cyclin D1, Cyclin D3, cdk4* have E boxes in their promoters that are recognized by BMAL1/CLOCK complex and show circadian oscillations in their expression (155,158).P21 is a cyclin-dependent kinase inhibitor that interacts with cdk2 complexes to regulate progression through G1. *Bmal1* deficiency was reported to up regulate expression of p21 in liver. Although no BMAL1/CLOCK responsive elements have been identified in the promoter region have been identified in the promoter region of p21, it might be indirectly regulated by BMAL1/CLOCK complex via RORs and REV-ERBs nuclear receptors (159,160).

Other groups have also demonstrated link between the clock proteins PERIODs and TIMELESS with cell cycle check point kinases ATM (Ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related protein). In response to DNA double strand breaks, ATM activates CHK2. Activated CHK2 inhibits activation of cdc2 required for cell cycle progression by inhibiting cdc25 phosphatase by phosphorylation. It was shown that PERIOD1 can interact with ATM/CHK2 complex and this interaction is important for activation of CHK2. Also,

TIM interacts with ATR/ATRIP (ATR interacting protein) and CHK1 kinase in response to stalled replication forks for G2/M arrest and the interaction is critical for CHK1 activation (161,162,163).

Based on our observations of increased proliferation of immortalized Bmal1-/- fibroblasts we hypothesized that *Bmal1* deficiency can result in deregulation of clock controlled cell cycle regulators that can result in change in progression though cell cycle for *Bmal1-/-* cells.To test this hypothesis we propose following experiments. We will perform cell cycle analysis of WT and *Bmal1-/-* immortalized lung fibroblasts using flow cytometry. This analysis will indicate relative number of cells in G1, S, G2 and M stage of cell cycle. The stage with least number of cells is likely the stage affected by Bmal1 deficiency. Depending on the number of cells per stage, we can assess expression of cell cycle regulators at mRNA level by Quantitative PCR and protein level by western blotting.

During our experiments, we also observed that *Bmal1* deficiency results in reduced sensitivity to certain anticancer drugs such as Etoposide and Daunorubicin. Based on our observations, we can hypothesize that the reduced sensitivity to Etoposide and Daunorubicin can be due to deregulation of cell cycle check points, we can perform cell cycle analysis. WT and *Bmal1-/-* cells treated with Etoposide and Daunorubicin can be analyzed for cell cycle distribution. The *Bmal1-/-* cells demonstrating higher number of cells in specific stage compared with WT suggests regulation by BMAL1.

4.2 Conclusions

Even after extensive work in the field of aging, mechanisms are poorly understood. Many studies with mutations of mTORC1 pathway components that either inhibit mTORC1 activity itself or its downstream effects have shown that inhibition of mTORC1 extends lifespan in various models from flies to mice. Treatment with Rapamycin (mTOR inhibitor) or calorie restriction (can affect mTORC1 activity) have been shown to extend life span in mice. This indicates that mTORC1 plays a significant role in aging.

Our results show that the circadian clock regulates mTORC1 activity through BMAL1 dependent mechanism and that increased mTORC1 activity can contribute to premature aging and age related pathologies in *Bmal1-/-* mice. Our findings suggest importance of appropriate function of circadian clock in organism's well-being and in aging with the fact that, we revealed two different ways (oxidative stress induced senescence and regulation of mTORC1 activity) through, which Clock is connected to aging. The research presented in this dissertation also indicates a vast unexplored area of mechanisms through that circadian clock is involved in aging.

REFERENCES

1. **Langmesser S, Albrecht U** Life time-circadian clocks, mitochondria and metabolism. Chronobiol Int. 2006; 23(1-2):151-7.
2. **Edery I** Circadian rhythms in a nutshell. Physiol Genomics. 2000 Aug 9;3(2):59-74
3. **Khapre RV Samsa WE, Kondratov RV** Circadian regulation of cell cycle: Molecular connections between aging and the circadian clock. Ann Med. 2010 Sep;42(6):404-15.**Khapre RV, Kondratova AA, Susova O, Kondratov RV**. Circadian clock protein BMAL1 regulates cellular senescence in vivo. Cell Cycle. 2011 Dec 1; 10(23):4162-9.
4. **Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA**.Mop3 is an essential component of the master circadian pacemaker in mammals. Cell. 2000 Dec 22; 103(7):1009-17.
5. **Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP.** Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. Genes Dev. 2006; 20:1868-1873.

6. **Hirayama J, Sassone-Corsi P** Structural and functional features of transcription factors controlling the circadian clock. Curr Opin Genet Dev. 2005 Oct; 15(5):548-56.

7. **Ko CH, Takahashi JS**. Molecular components of the mammalian circadian clock. Hum Mol Genet. 2006 Oct 15;15 Spec No 2:R271-7

8. **Kazuhiko Kume, Mark J Zylka Sathyanarayanan Sriram, Lauren P Shearman, David R Weaver, Xiaowei Jin, Elizabeth S Maywood, Michael H Hastings, Steven M Reppert** mCRY1 and mCRY2 Are Essential Components of the Negative Limb of the Circadian Clock Feedback Loop Cell, 23 July 1999, Pages 193–205

9. **Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M, Kato Y, Honma K.** Dec1 and Dec2 are regulators of the mammalian molecular clock. Nature. 2002 Oct 24; 419(6909):841-4.

10. **Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, Naik KA, FitzGerald GA, Kay SA, Hogenesch JB.** A functional genomics strategy reveals Rora as a component of the mammalian circadian clock Neuron. 2004 Aug 19; 43(4):527-37.

11. **Hiroki R. Ueda, Wenbin Chen, Akihito Adachi, Hisanori Wakamatsu, Satoko Hayashi, Tomohiro Takasugi, Mamoru Nagano, Ken-ichi Nakahama, Yutaka Suzuki, Sumio Sugano, Masamitsu Iino, Yasufumi Shigeyoshi & Seiichi Hashimoto** A transcription factor response element for gene expression during circadian night Nature August 2002 418, 534-539

12. **Nicolas Preitner**, **Francesca Damiola**, **Luis-Lopez-Molina**, **Joszef Zakany**, **Denis Duboule**, **Urs Albrecht and Ueli Schibler** The Orphan Nuclear Receptor REV-ERB Controls Circadian Transcription within the Positive Limb of the Mammalian Circadian Oscillator Cell, 2002 251–260

13. **Zhihua Liu, Guiyan Chu** Chronobiology in mammalian health Molecular Biology Reports March 2013, pp 2491-2501

14. **Joseph S. Takahashi, Hee-Kyung Hong, Caroline H. Ko & Erin L. McDearmo** The genetics of mammalian circadian order and disorder: implications for physiology and disease Nature Reviews Genetics 9 October 2008, 764-775

15. **Fabian Preuss, Yueming Tang, Aaron D. Laposky, Deanna Arble, Ali Keshavarzian, Fred W. Turek** Adverse effects of chronic circadian desynchronization in animals in a “challenging” environment Am J Physiol Regul Integr Comp Physiol. 2008 December; 295(6): R2034–R2040

16. **Masako Okawa,Makoto Uchiyama Circadian rhythm sleep disorders**:Characteristics and entrainment pathology in delayed sleep phase and non-24 sleep wake syndrome Sleep Medicine Reviews (2007) 11, 485–496

17. **Arble DM, Ramsey KM, Bass J, Turek FW**.Circadian disruption and metabolic disease: findings from animal models. Best Pract Res Clin Endocrinol Metab. 2010 Oct;24(5):785-800

18. **Kondratov RV**. A role of the circadian system and circadian proteins in aging. Ageing Res Rev. 2007 May; 6(1):12-27.

19. **Elizabeth A. Yu and David R. Weave** Disrupting the circadian clock: Gene-specific effects on aging, cancer, and other phenotypes Aging (Albany NY). 2011 May; 3(5): 479–493

20. **Kondratov RV, Vykhovanets O, Kondratova AA, Antoch, MP**. Antioxidant N-acetyl-L-cysteine ameliorates symptoms of premature aging associated with the deficiency of the circadian protein BMAL1. Aging (Albany) 2009; 1 :979-987

21. **Boden MJ, Varcoe TJ, Voultsios A, Kennaway DJ.** Reproductive biology of female Bmal1 null mice. Reproduction. 2010; 139:1077-1090.

22. **Sun Y, Yang Z, Niu Z, Wang W, Peng J, Li Q, Ma MY, Zhao Y.** The mortality of MOP3 deficient mice with a systemic functional failure. J Biomed Sci. 2006; 13:845-51.

23. **A Klarsfeld, F Rouyer** Effects of circadian mutations and LD periodicity on the life span of Drosophila melanogaster. Journal of Biological Rhythms 01/1999; 13(6):471-8

24. **Kiho Bae, Xiaowei Jin, Elizabeth S. Maywood, Michael H. Hastings, Steven M. Reppert, David R. Weaver** Differential Functions of *mPer1*, *mPer2*, and *mPer3* in the SCN Circadian Clock Neuron May 2001, Pages 525–536

25. **Zheng et al., 2001, B. Zheng, U. Albrecht, K. Kaasik, M. Sage, W. Lu, S. Vaishnav, Q. Li, Z.S. Sun, G. Eichele, A. Bradley, C.C**. Lee Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock Cell, 105 (2001), pp. 683–694

26. **Hua H, Wang Y, Wan C, Liu Y, Zhu B, Wang X, Wang Z, Ding JM**. Inhibition of tumorigenesis by intratumoral delivery of the circadian gene mPer2 in C57BL/6 mice. Cancer Gene Ther. 2007; 14:815-818

27. **Hua H, Wang Y, Wan C, Liu Y, Zhu B, Yang C, Wang X, Wang Z, Cornelissen-Guillaume G, Halberg F**. Circadian gene *mPer2* overexpression induces cancer cell apoptosis. Cancer Sci. 2006; 97:589-96

28. **C.C. Lee** The circadian clock and tumor suppression by mammalian period genes Meth. Enzymol., 393 (2005), pp. 852–861

29. **Oda A, Katayose Y, Yabuuchi S, Yamamoto K, Mizuma M, Shirasou S, Onogawa T, Ohtsuka H, Yoshida H, Hayashi H, Rikiyama T, Kim H, Choe Y** Clock gene mouse period2 overexpression inhibits growth of human pancreatic cancer cells and has synergistic effect with cisplatin. Anticancer Res. 2009; 29:1201-1209.

30. **Gauger MA, Sancar A.** Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer. Cancer Res. 2005; 65:6828-6834.

31. **Lee S, Donehower LA, Herron AJ, Moore DD, Fu L.** Disrupting circadian homeostasis of sympathetic signaling promotes tumor development in mice. PLoS One. 2010; 5:e10995.

32. **Zturk N, Lee JH, Gaddameedhi S, Sancar A**. Loss of cryptochrome reduces cancer risk in p53 mutant mice. Proc Natl Acad Sci U S A. 2009; 106:2841-284

33. **Dubrovsky YV, Samsa WE, Kondratov RV**.Deficiency of circadian protein CLOCK reduces lifespan and increases age-related cataract development in mice. Aging (Albany NY). 2010 Dec; 2(12):936-44.

34. **Carol A. Dudley, Claudia Erbel-Sieler, Sandi Jo Estill, Martin Reick, Paul Franken, SiNae Pitts, Steven L. McKnight** Altered Patterns of Sleep and Behavioral Adaptability in NPAS2-Deficient Mice Science 2003 no. 5631 pp. 379-383

35. **Jason P DeBruyne, David R Weaver & Steven M Reppert** CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock Nature Neuroscience 10, 543 - 545 (2007)

36. **DeBruyne JP, Weaver DR, Reppert SM** Peripheral circadian oscillators require CLOCK. Curr Biol. 2007 Jul 17; 17(14):R538-9.

37. **Dolatshad H, Campbell EA, O'Hara L, Maywood ES, Hastings MH, Johnson MH.** Developmental and reproductive performance in circadian mutant mice. Hum Reprod. 2006; 21:68-79

38. **Kennaway DJ, Boden MJ, Voultsios A.** Reproductive performance in female clock Delta19 mutant mice. Reprod Fertil Dev. 2004; 16:801-810.

39. **Antoch MP, Gorbacheva VY, Vykhovanets O, Toshkov IA, Kondratov RV, Kondratova AA, Lee C, Nikitin AY**. Disruption of the circadian clock due to the Clock mutation has discrete effects on aging and carcinogenesis. Cell Cycle. 2008; 7:1197-1204.

40. **Campisi J.** Senescent cells, tumor suppression and organismal aging: good citizens, bad neighbors. Cell 2005; 120:513-22; PMID: 15734683; DOI: 10.1016/j. cell.2005.02.003.

41. **Wang Y, Liang Y, Vanhoutte PM**.SIRT1 and AMPK in regulating mammalian senescence: a critical review and a working model. FEBS Lett. 2011 Apr 6; 585(7):986-94.

42. **Hayflick L.** The Limited in Vitro Lifetime of Human Diploid Cell Strains. Exp Cell Res 1965; 37:614-36 PMID:14315085; DOI:10.1016/0014-4827(65)90211-9

43. **Cristofalo VJ, Lorenzini A, Allen RG, Torres C, Tresini M** Replicative senescence: a critical review. Mech Ageing Dev. 2004 Oct-Nov;125(10-11):827-48

44. **U. Brunk, J.L.E. Ericsson, J. Pontén,B.** Westermark Residual bodies and “aging” in cultured human glia cells : Effect of entrance into phase III and prolonged periods of confluence Experimental Cell research May 1973, Pages 1–14

45. **Rheinwald, J.G.a.G.H**. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 1975., 331–334

46. **E.L. Bierman** The effects of donor age on the in vitro life span of cultured human arterial smooth-muscle cells In Vitro, 14 (1978), pp. 951–955

47. **J. Tassin, E. Malaise, Y. Courtois** Human lens cells have an in vitro proliferative capacity inversely proportional to the donor age Exp. Cell Res., 123 (1979), pp. 388–392

48. **S.N. Mueller, E.M. Rose, E.M. Levine** Cellular senescence in a cloned strain of bovine fetal aortic endothelial cells Science, 207 (1980), pp. 889–891

49. **R.R. Tice, E.L. Schneider, D. Kram, P. Thorne** Cytokinetic analysis of impaired proliferative response of peripheral lymphocytes from aged humans to phytohemagglutinin Exp. Med., 149 (1979), pp. 1029–1041

50. **Campisi J.Aging**, cellular senescence, and cancer. Annu Rev Physiol. 2013;75:685-705

51. **Jeyapalan JC, Sedivy JM.**Cellular senescence and organismal aging. Mech Ageing Dev. 2008 Jul-Aug; 129(7-8):467-74.

52. **Marcotte R, Lacelle C, Wang E** Senescent fibroblasts resist apoptosis by downregulating caspase-3. Mech Ageing Dev. 2004 Oct-Nov; 125(10-11):777-83.

53. **Le Guilly, M. Simon, P. Lenoir, M. Bourel** Long-term culture of human adult liver cells: morphological changes related to in vitro senescence and effect of donor's age on growth potential Gerontologia, 19 (1973), pp. 303–313

54. **G.M. Martin, C.A. Sprague, C.J. Epstein** Replicative life-span of cultivated human cells. Effects of donor's age, tissue, and genotype Lab. Invest., 23 (1970), pp. 86–92

55. **Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, et al.** A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 1995; 92:9363-7; PMID: 7568133; DOI:10.1073/pnas.92.20.9363. 27.

56. **Shelton DN, Chang E, Whittier PS, Choi D, Funk WD.** Microarray analysis of replicative senescence. Curr Biol 1999; 9:939-45; PMID: 10508581; DOI: 10.1016/S0960-9822(99)80420-5. 28

57. **Joa o Pedro de Magalhaes** From cells to ageing: a review of models and mechanisms of cellular senescence and their impact on human ageing Experimental Cell Research 300 (2004) 1–10

58. **Coppé JP, Patil CK, Rodier F, Sun Y, Munoz DP, Goldstein J**. Senescence-associated secretory phe- notypes reveal cell-nonautonomous functions of onco- genic RAS and the p53 tumor suppressor. PLoS Biol 2008; 6:2853-68;

59. **Faragher RG, Kipling D**. How might replicative senescence contribute to human ageing? Bioessays 1998; 20:985-91

60. **Wang C, Jurk D, Maddick M, Nelson G, Martin-Ruiz C, von Zglinicki T.** DNA damage response and cellular senescence in tissues of aging mice. Aging Cell 2009; 8:311-23;

61. **Smith JR, Pereira-Smith OM.** Replicative senescence: implications for in vivo aging and tumor suppres- sion. Science 1996; 273:63-7;

62. **Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW**. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997; 88:593-602

63. **Robles SJ, Adami GR**. Agents that cause DNA double strand breaks lead to p16INK4a enrichment and the premature senescence of normal fibroblasts. Oncogene 1998; 16:1113-23;

64. **Chen Q, Fischer A, Reagan JD, Yan LJ, Ames BN.** Oxidative DNA damage and senescence of human diploid fibroblast cells. Proc Natl Acad Sci USA 1995; 92:4337-41;

65. **Blagosklonny MV. Aging**: ROS or TOR. Cell Cycle 2008; 7:3344-54; PMID: 18971624; DOI:10.4161/ cc.7.21.6965.

66. **Brown-Borg HM.** Hormonal regulation of longev- ity in mammals. Ageing Res Rev 2007; 6:28-45; PMID:17360245; DOI:10.1016/j.arr.2007.02.005

67. **Crespo JL, Hall MN** Elucidating TOR Signaling and Rapamycin Action: Lessons from *Saccharomyces cerevisiae.* Microbiol Mol Biol Rev. 2002 December; 66(4): 579–591.

68. **Vézina C, Kudelski A, Sehgal SN.**Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. Antibiot (Tokyo). 1975 Oct;28(10):721-6.

69. **Watanabe R, Wei L, Huang J.**mTOR signaling, function, novel inhibitors, and therapeutic targets. J Nucl Med. 2011 Apr;52(4):497-500

70. **Wang X, Proud CG**.mTORC1 signaling: what we still don't know. J Mol Cell Biol. 2011 Aug;3(4):206-20

71. **Hay N, Sonenberg N**.Upstream and downstream of mTOR. Genes Dev. 2004 Aug 15;18(16):1926-45

72. **David A. Guertin, David M. Sabatini** Defining the Role of mTOR in Cancer Cancer Cell July 2007, Pages 9–22

73. **Huang J, Manning BD** The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. Biochem J. 2008 Jun 1; 412(2):179-90.

74. **Dan HC, Sun M, Yang L, Feldman RI, Sui XM, Ou CC, Nellist M, Yeung RS, Halley DJ, Nicosia SV, Pledger WJ, Cheng JQ**.Phosphatidylinositol 3-kinase/Akt pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberin. J Biol Chem. 2002 Sep 20;277(38):35364-70

75. **Manning BD, Cantley LC** AKT/PKB signaling: navigating downstream. Cell. 2007 Jun 29;129(7):1261-74.

76. **Burnett PE, Barrow RK, Cohen NA, Snyder SH, Sabatini DM.**RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1Biochemistry Vol. 95, pp. 1432–1437, February 1998

77. **Law BK**.Rapamycin: an anti-cancer immunosuppressant? Crit Rev Oncol Hematol.2005 Oct;56(1):47-60.

78. **Mita MM, Mita A, Rowinsky EK**.Mammalian target of rapamycin: a new molecular target for breast cancer. Clin Breast Cancer. 2003 Jun;4(2):126-37

79. **Mita MM, Mita A, Rowinsky EK**.The molecular target of rapamycin (mTOR) as a therapeutic target against cancer. 2003 Jul-Aug;2(4 Suppl 1):S169-77.

80. **Um SH, D'Alessio D, Thomas G** Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. Cell Metab 2006 Jun;3(6):393-402.

81. **Zoncu R, Efeyan A, Sabatini DM** mTOR: from growth signal integration to cancer, diabetes and ageingNat Rev Mol Cell Biol. 2011 Jan;12(1):21-35.

82. **Dann SG, Selvaraj A, Thomas G** mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. rends Mol Med. 2007 Jun;13(6):252-9.

83. **Shima H, Pende M,Chen Y, Fumagalli S, Thomas G, Kozma SC** Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. EMBO J 1998;17(22):6649-6659

84. **Pende M, Kozma SC, Jaquet M, Oorschot V, Burcelin R, Le Marchand-Brustel Y, Klumperman J, Thorens B, Thomas G** Hypoinsulinaemia, glucose intolerance and diminished beta-cell size in S6K1-deficient mice. Nature. 2000 Dec 21-28;408(6815):994-7.

85. **Castilho RM, Squarize CH, Chodosh LA, Williams BO, Gutkind** JS mTOR mediates Wnt-induced epidermal stem cell exhaustion and aging. Cell Stem Cell. 2009 Sep 4;5(3):279-89.

86. **Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Müller F**. Influence of TOR kinase on lifespan in C. elegans. Genetics:2003 Dec 11;426(6967):620.

87. **Pankaj Kapahi, Brian M. Zid, Tony Harper, Daniel Koslover, Viveca Sapin, and Seymour Benzer**. Regulation of Lifespan in *Drosophila* by Modulation of Genes in the TOR Signaling Pathway Curr Biol. 2004 May 25; 14(10): 885–890.

88. **Powers RW 3rd, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S**.Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes Dev. 2006 Jan 15;20(2):174-84

89. **Ching-Chyuan Hsieh, John Papaconstantinou** Akt/PKB and p38 MAPK signaling, translational initiation and longevity in Snell dwarf mouse livers Mech Ageing Dev. 2004 Oct-Nov;125(10-11):785-98

90. **Selman C, Tullet JM, Wieser D, Irvine E, Lingard SJ, Choudhury AI, Claret M, Al-Qassab H, Carmignac D, Ramadani F, Woods A, Robinson IC, Schuster E, Batterham RL, Kozma SC, Thomas G, Carling D, Okkenhaug K, Thornton JM, Partridge L, Gems D, Withers DJ** Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. Science. 2009 Oct 2;326(5949):140-4

91. **Zid BM, Rogers AN, Katewa SD, Vargas MA, Kolipinski MC, Lu TA, Benzer S, Kapahi** P 4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila. Cell. 2009 Oct 2;139(1):149-60.

92. **Alarcon, C. M., J. Heitman, and M. E. Cardenas.** 1999. Protein kinase activity and identification of a toxic effector domain of the target of rapamycin TOR proteins in yeast. Mol. Biol. Cell 10**:**2531-2546.

93. **Helliwell, S. B., P. Wagner, J. Kunz, M. Deuter-Reinhard, R. Henriquez, and M. N. Hall.** 1994. TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. Mol. Biol. Cell 5**:**105-118

94. **Kunz, J., R. Henriquez, U. Schneider, M. Deuter-Reinhard, N. R. Movva, and M. N. Hall.** 1993. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G_1 progression. Cell 73**:**585-596

95. **Alarcon, C. M., J. Heitman, and M. E. Cardenas.** 1999. Protein kinase activity and identification of a toxic effector domain of the target of rapamycin TOR proteins in yeast. Mol. Biol. Cell 10**:**2531-2546

96. **Andrade MA, Bork P** HEAT repeats in the Huntington's disease protein. Nat Genet. 1995 Oct;11(2):115-6.

97. **Yang H, Rudge DG, Koos JD, Vaidialingam B, Yang HJ, Pavletich NP** mTOR kinase structure, mechanism and regulation. Nature. 2013 May 9;497(7448):217-23

98. **Choi, J., J. Chen, S. L. Schreiber, and J. Clardy.** 1996. Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. Science 273**:**239-242.

99. **Bosotti, R., A. Isacchi, and E. L. Sonnhammer.** 2000. FAT: a novel domain in PIK-related kinases. Trends Biochem. Sci. 25**:**225-227.

100. **Watanabe R, Wei L, Huang J** mTOR signaling, function, novel inhibitors, and therapeutic targets. J Nucl Med. 2011 Apr;52(4):497-500

101. **Wang X, Proud CG.** mTORC1 signaling: what we still don't know. J Mol Cell Biol. 2011 Aug;3(4):206-20

102. **Hay N, Sonenberg N**.Upstream and downstream of mTOR. Genes Dev. 2004 Aug 15;18(16):1926-45.

103. **Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, Tokunaga C, Avruch J, Yonezawa K**.Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell. 2002 Jul 26;110(2):177-89.

104. **Kim DH, Sarbassov DD, Ali SM, Latek RR, Guntur KV, Erdjument-Bromage H, Tempst P, Sabatini DM**. GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol Cell. 2003 Apr;11(4):895-904.

105. **Wang H, Zhang Q, Wen Q, Zheng Y, Lazarovici P, Jiang H, Lin J, Zheng W**.Proline-rich Akt substrate of 40kDa (PRAS40): a novel downstream target of PI3k/Akt signaling pathway. Cell Signal. 2012 Jan;24(1):17-24.

106. **Peterson TR, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, Gray NS, Sabatini DM** DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell. 2009 May 29;137(5):873-86

107. **Gao D, Inuzuka H, Tan MK, Fukushima H, Locasale JW, Liu P, Wan L, Zhai B, Chin YR, Shaik S, Lyssiotis CA, Gygi SP, Toker A, Cantley LC, Asara JM, Harper JW, Wei W.** mTOR Drives Its Own Activation via $SCF^{\beta TrCP}$-Dependent Degradation of the mTOR Inhibitor DEPTOR Mol Cell. 2011 Oct 21;44(2):290-303

108. **Ruvinsky I, Sharon N, Lerer T, Cohen H, Stolovich-Rain M, Nir T, Dor Y, Zisman P, Meyuhas O** Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. Genes Dev. 2005 Sep 15;19(18):2199-211

109. ***vivo*Eric J. Brown, Peter A. Beal, Curtis T. Keith, Jie Chen, Tae Bum Shin & Stuart L. Schreiber** Control of p70 S6 kinase by kinase activity of FRAP in Nature 377, 441-446 (5 October 1995)

110. **Hara K, Yonezawa K, Kozlowski MT, Sugimoto T, Andrabi K, Weng QP, Kasuga M, Nishimoto I, Avruch J**.Regulation of eIF-4E BP1 phosphorylation by mTOR. J Biol Chem. 1997 Oct 17;272(42):26457-63.

111. **Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J**.Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. J Biol Chem. 1998 Jun 5;273(23):14484-94.

112. **Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM** Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol. 2004 Jul 27;14(14):1296-302

113. **Pearce LR, Huang X, Boudeau J, Pawłowski R, Wullschleger S, Deak M, Ibrahim AF, Gourlay R, Magnuson MA, Alessi DR**.Identification of Protor as a novel Rictor-binding component of mTOR complex-2. Biochem J. 2007 Aug 1;405(3):513-22

114. **Yang Q, Inoki K, Ikenoue T, Guan KL**.Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. Genes Dev. 2006 Oct 15;20(20):2820-32

115. **Sengupta S, Peterson TR, Sabatini DM**.Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Mol Cell. 2010 Oct 22;40(2):310-22.

116. **Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM** The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science. 2008 Jun 13;320(5882):1496-501

117. **Sancak Y, Sabatini DM** Rag proteins regulate amino-acid-induced mTORC1 signalling. Biochem Soc Trans. 2009 Feb;37(Pt 1):289-90

118. **Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM**.Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell. 2010 Apr 16;141(2):290-303

119. **Bar-Peled L, Schweitzer LD, Zoncu R, Sabatini DM** Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. Cell. 2012 Sep 14;150(6):1196-208.

120. **Long X, Lin Y, Ortiz-Vega S, Yonezawa K, Avruch J** Rheb binds and regulates the mTOR kinase. Curr Biol. 2005 Apr 26;15(8):702-13.

121. **Inoki K, Li Y, Xu T, Guan KL.**Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev. 2003 Aug 1;17(15):1829-34.

122. **Huang J, Manning BD** The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. Biochem J. 2008 Jun 1;412(2):179-90.

123. **Tee AR, Fingar DC, Manning BD, Kwiatkowski DJ, Cantley LC, Blenis J** Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. Proc Natl Acad Sci U S A. 2002 Oct 15;99(21):13571-6

124. **Inoki K, Li Y, Xu T, Guan KL**.Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev. 2003 Aug 1;17(15):1829-34.

125. **Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J**.Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol. 2003 Aug 5;13(15):1259-68

126. **Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, Kozma SC, Hafen E, Bos JL, Thomas G.**Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell. 2003 Jun;11(6):1457-66

127. **Inoki K, Li Y, Zhu T, Wu J, Guan KL.**TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol. 2002 Sep;4(9):648-57

128. **Yamagata K, Sanders LK, Kaufmann WE, Yee W, Barnes CA, Nathans D, Worley PF**.rheb, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein. J Biol Chem. 1994 Jun 10;269(23):16333-9

129. **Avruch J, Long X, Lin Y, Ortiz-Vega S, Rapley J, Papageorgiou A, Oshiro N, Kikkawa U.**Activation of mTORC1 in two steps: Rheb-GTP activation of catalytic function and increased binding of substrates to raptor. Biochem Soc Trans. 2009 Feb;37(Pt 1):223-6.

130. **Nellist M, Goedbloed MA, de Winter C, Verhaaf B, Jankie A, Reuser AJ, van den Ouweland AM, van der Sluijs P, Halley DJ.** Identification and characterization of the interaction between tuberin and 14-3-3zeta. J Biol Chem. 2002 Oct 18;277(42):39417-24

131. **Nellist M, Goedbloed MA, Halley DJ** Regulation of tuberous sclerosis complex (TSC) function by 14-3-3 proteins. Biochem Soc Trans. 2003 Jun;31(Pt 3):587-91.

132. **Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, Carr SA, Sabatini DM.**PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol Cell. 2007 Mar 23;25(6):903-15

133. **Y. Fang, M. Vilella-Bach, R. Bachmann, A. Flanigan, J.** Chen Phosphatidic acid-mediated mitogenic activation of mTOR signaling Science, 294 (2001), pp. 1942–1945

134. **D.A. Foster Phosphatidic acid signaling to mTOR:** signals for the survival of human cancer cells Biochim. Biophys. Acta, 1791 (2009), pp. 949–955

135. **M.N. Corradetti, K. Inoki, N. Bardeesy, R.A. DePinho, K.L.** Guan Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome Genes Dev. 2004 Jul 1;18(13):1533-8

136. **R.J. Shaw** LKB1 and AMP-activated protein kinase control of mTOR signalling and growth Acta Physiol. (Oxf.), 196 (2009), pp. 65–80

137. **Inoki K, Ouyang H, Zhu T, Lindvall C, Wang Y, Zhang X, Yang Q, Bennett C, Harada Y, Stankunas K, Wang CY, He X, MacDougald OA, You M, Williams BO, Guan KL.** TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. Cell. 2006 Sep 8;126(5):955-68

138. **D.M. Gwinn, D.B. Shackelford, D.F. Egan, M.M. Mihaylova, A. Mery, D.S. Vasquez, B.E. Turk, R.J. Shaw** AMPK phosphorylation of raptor mediates a metabolic checkpoint Mol. Cell, 30 (2008), pp. 214–226

139. **J. Brugarolas, K. Lei, R.L. Hurley, B.D. Manning, J.H. Reiling, E. Hafen, L.A. Witters, L.W. Ellisen, W.G. Kaelin Jr.** Regulation of mTOR function

in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex Genes Dev. 2004 Dec 1;18(23):2893-904

140. **DeYoung MP, Horak P, Sofer A, Sgroi D, Ellisen LW** Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. Genes Dev. 2008 Jan 15;22(2):239-51

141. **Ronald J. Konopka, Seymour Benzer** Clock mutants of Drosophila MelanogasterProc.Nat.Acad.Sci.USA1971 Sep;68(9):2112-2116

142. **Faraut B, Bayon V, Léger D** Neuroendocrine, immune and oxidative stress in shift workers Sleep Med Rev. 2013 Dec;17(6):433-44.

143. **Zeng ZL1, Wu MW, Sun J, Sun YL, Cai YC, Huang YJ, Xian LJ** Effects of the biological clock gene Bmal1 on tumour growth and anti-cancer drug activity. J Biochem. 2010 Sep;148(3):319-26

144. **Mullenders J, Fabius AW, Madiredjo M, Bernards R, Beijersbergen RL** A large scale shRNA barcode screen identifies the circadian clock component ARNTL as putative regulator of the p53 tumor suppressor pathway. PLoS One 2009 4(3):4798

145. **Musiek ES, Lim MM, Yang G, Bauer AQ, Qi L, Lee Y, Roh JH, Ortiz-Gonzalez X, Dearborn JT, Culver JP, Herzog ED, Hogenesch JB, Wozniak DF, Dikranian K, Giasson BI, Weaver DR, Holtzman DM, Fitzgerald GA.** Circadian clock proteins regulate neuronal redox homeostasis and neurodegeneration. J Clin Invest. 2013 Dec 2;123(12):5389-400

146. **Pause A1, Belsham GJ, Gingras AC, Donzé O, Lin TA, Lawrence JC Jr, Sonenberg N.** Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. Nature. 1994 Oct 27;371(6500):762-7

147. **Richardson CJ1, Bröenstrup M, Fingar DC, Jülich K, Ballif BA, Gygi S, Blenis J** SKAR is a specific target of S6 kinase 1 in cell growth control. Curr Biol. 2004 Sep 7;14(17):1540-9

148. **Raught B., Peiretti, F., Gingras, A.C., Livingstone, M., Shahbazian, D., Mayeur, G.L., Polakiewicz, R.D., Sonenberg, N., and Hershey**, **J.W.** Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. 2004 EMBO J. 23: 1761-1769

149. **Dorrello NV1, Peschiaroli A, Guardavaccaro D, Colburn NH, Sherman NE, Pagano M.** S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science. 2006 Oct 20;314(5798):467-71

150. **Holz MK1, Ballif BA, Gygi SP, Blenis J**. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. Cell. 2005 Nov 18;123(4):569-80

151. **Peterson TR1, Sabatini DM** eIF3: a connecTOR of S6K1 to the translation preinitiation complex. Mol Cell. 2005 Dec 9;20(5):655-7

152. **Vermeulen K1, Van Bockstaele DR, Berneman ZN** The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer Cell Prolif. 2003 Jun;36(3):131-49.

153. **Malumbres M** Revisiting the "Cdk-centric" view of the mammalian cell cycle. Cell Cycle. 2005 Feb;4(2):206-10.

154. **Edmunds LN Jr, Funch RR.** Circadian rhythm of cell division in Euglena: effects of random illumination regimen. Science. 1969 Aug 1;165(3892):500-3.

155. **Fu L1, Pelicano H, Liu J, Huang P, Lee C.** The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. Cell. 2002 Oct 4;111(1):41-50

156. **Barsyte-Lovejoy D1, Mao DY, Penn LZ** c-Myc represses the proximal promoters of GADD45a and GADD153 by a post-RNA polymerase II recruitment mechanism Oncogene. 2004 Apr 22;23(19):3481-6.

157. **Matsuo T1, Yamaguchi S, Mitsui S, Emi A, Shimoda F, Okamura H**. Control mechanism of the circadian clock for timing of cell division in vivo. Science. 2003 Oct 10;302(5643):255-9

158. **Grundschober C1, Delaunay F, Pühlhofer A, Triqueneaux G, Laudet V, Bartfai T, Nef P**. Circadian regulation of diverse gene products revealed by mRNA expression profiling of synchronized fibroblasts. J Biol Chem. 2001 Dec 14;276(50):46751-8.

159. **Gréchez-Cassiau A1, Rayet B, Guillaumond F, Teboul M, Delaunay** The circadian clock component BMAL1 is a critical regulator of

p21WAF1/CIP1 expression and hepatocyte proliferation. J Biol Chem. 2008 Feb 22;283(8):4535-42

160. **Deng C1, Zhang P, Harper JW, Elledge SJ, Leder P.** Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell. 1995 Aug 25;82(4):675-84

161. **Kondratov RV1, Gorbacheva VY, Antoch MP**. The role of mammalian circadian proteins in normal physiology and genotoxic stress responses Curr Top Dev Biol. 2007;78:173-216

162. **Gery S1, Komatsu N, Baldjyan L, Yu A, Koo D, Koeffler HP**. The circadian gene per1 plays an important role in cell growth and DNA damage control in human cancer cells. Mol Cell. 2006 May 5;22(3):375-82

163. **Unsal-Kaçmaz K1, Mullen TE, Kaufmann WK, Sancar A**. Coupling of human circadian and cell cycles by the timeless protein. Mol Cell Biol. 2005 Apr;25(8):3109-16